Emile Jonker

Vaccination

Intradermal administration, duration of protection, and compromised immunity

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Cover page illustration: "L'origine de la vaccine". A milk maid shows her cowpoxed hand to a physician, while a farmer or surgeon offers to a dandy inoculation with cowpox that he has taken from a cow. Coloured etching, ca. 1800. Wellcome Collection. Public Domain Mark.

Vaccination: intradermal administration, duration of protection, and compromised immunity

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Chapter 1

Introduction and outline of the thesis

1.1 Introduction

Vaccination is one of the most effective contributions to population health in history (1), after the introduction of general hygiene measures to prevent disease. Diseases that once were prevalent in society and led to high morbidity among children have been virtually eliminated through national immunization programs, although outbreaks persist in areas with reduced vaccination rates (2). The pivotal importance of vaccination is again strikingly evident in the current COVID-19 pandemic.

Vaccination is also an effective tool to protect travelers against the infectious risks inherent to travel (3). Some of these risks can be mitigated by changing behavior through education (4) but reducing vulnerability through vaccination is essential (5). Although the research in this thesis was conceived in the context of travelers' vaccination, we hope that the data generated benefits the local populations that are most affected by these diseases.

Despite wide availability in high income countries, not every traveler seeks pre-travel health advice and vaccination. Several known causes are: insufficient knowledge about the necessity and availability of such advice, insufficient time before departure, and high costs of vaccination (6). In addition, there has been a notable increase in vaccine shortages in recent years, which also affected travelers preparing for their travels (7) (8). Vaccine shortages are usually caused by setbacks in the production process, which for most vaccines is more cumbersome and vulnerable than it is for other types of medication. It can also take significantly more time to ramp up production in response to fluctuating demands (personal communication).

Traditionally, vaccines are administered into the muscle (intramuscular administration, IM). However, the skin (dermis) contains a much higher density of antigen presenting dendritic cells than the muscle (9). The skin lymphatic system is extensively organized into several plexus systems, which aids efficient transport of antigen presenting dendritic cells to the regional lymph nodes (10). As a consequence, a lower vaccine dose introduced directly into the dermis (intradermal administration, ID) as opposed to the muscle (intramuscular administration, IM) will often be sufficient to achieve a protective immune response.

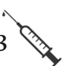
A recent systemic review and meta-analysis showed that fractional ID administration is non-inferior to IM administration for influenza, rabies and hepatitis B vaccination (the latter only in ID doses above 2 micrograms) (11). Both intradermal vaccination for rabies and seasonal influenza has

been endorsed by WHO since 2011 (12). For yellow fever, polio, hepatitis A, diptheria tetanus and pertussis (DTP), human papillomavirus, Japanese encephalitis and varicella zoster, the number of studies was limited and did not fulfill the criteria for inclusion in a meta-analysis, but results are promising. For measles, ID administration was inferior to IM in the majority of studies performed in healthy adults. A selected overview of the studies mentioned above (extracted from Schnyder et al (11)), limited to healthy adults and comparing fractional ID dose against IM dosing in WHO recommended dosages is summarized in table 1.

Apart from the reduction in amount of vaccine used by fractional (ID) dosing, further reductions are possible by reducing the number of injections in the primary or revaccination immunization schedule. Regardless of the strategy chosen, some vaccines are more amenable to these modifications than others, depending on their intrinsic immunogenicity or excess of antigen present in a standard dose.

Table 1: Studies comparing fractional dose ID to standard dose IM of SC administration, using WHO approved immunization schedules for registered vaccines, in healthy adults.

	Number of studies	ID superior	ID equivalent	ID inferior
Influenza (IM)	16	0	16	0
Hepatitis B (IM)	13	0	7	6
Rabies (prep) (IM)	24	0	21	3
Inactivated Polio Virus (3 doses) (IM)	3	0	2	1
Measles (IM or SC)	6	0	2	4
Hepatitis A (IM)	4	0	3	1
Diphtheria/tetanus/pertusis (IM)	2	0	2	0
Human papillomavirus (IM)	1	0	1	0
Japanese encephalitis (IM)	2	0	2	0
Meningococcal disease (SC)	0	0	0	0
Varicella zoster (SC)	1	0	1	0
Yellow fever (SC)	1	0	1	0



1.2 Outline of this thesis

The first part of this thesis will focus on dose-sparing strategies. We evaluated the available evidence and investigated the efficacy of dose reduction (either through fractional dosing or eliminating doses from the vaccination schedule) for vaccines against 3 infections that have seen recent or recurrent outbreaks and for which vaccine shortages regularly occur, either due to production issues, costs or a sudden increase in demand. The diseases in question are rabies (**Chapter 2**), meningococcal disease (**Chapter 3**) and yellow fever (**Chapter 5**), each of which is discussed in more detail below.

The Zero by 30 campaign gained traction in 2016 to prevent human deaths from rabies exposures by 2030 (13) by ensuring equitable, affordable and timely access to health care, medicines and vaccines. It was recognized that canine vaccination is the most cost-effective avenue of global reduction of human rabies through dog bites (14). Although rabies can be transmitted by all mammals (bats and South-East Asian monkeys come to mind as the source of many exposures), our focus is on human vaccination.

Looking at published data, not only from research focused on pre-exposure prophylaxis but also looking at applicable time points from post-exposure prophylaxis studies in rabies-naive subjects, we hypothesized that extensive dose reduction was possible. From these data we hypothesized that a single rabies vaccine fractional dose may be sufficient to prime the immune system in such a way that it will result in a fast memory response after revaccination, even in the absence of seroconversion after primary vaccination.

In **Chapter 2** we explored this hypothesis in a dose-finding study in healthy volunteers in which we reduced the standard intramuscular dose for pre-exposure prophylaxis up to 15 times to an equivalent of 6,7% in terms of standard antigen exposure.

Spurred on by the success of the conjugate meningococcal A mass vaccination campaigns, and because of the changing epidemiology of meningococcal disease in Africa from serogroup A to serogroup W and the emergence of serogroup W outbreaks across Europe (15), we performed a dose-escalation study with two quadrivalent conjugated meningococcal vaccines against serogroups A, C, Y, and W (MenACWY-CRM197 Menveo® and MenACWY-TT Nimenrix ®) in **Chapter 3**. Conjugated meningococcal vaccines are costly and short in supply due to the high demand resulting from the recent inclusion of these vaccines into national immunization programs.

Besides limited availability and high costs, other factors may contribute to less-than-desirable protection. In case of immune suppression, the patient not only becomes more vulnerable to various infectious diseases, the effectiveness of the body's response to vaccines may be severely reduced (16) (17) (18). In case of live-attenuated vaccines, severe immune suppression is an absolute contraindication against vaccination (5).

In the second part of this thesis we address some of the questions regarding the effect of different immune suppressive regimes on vaccine responses and long-term immunity after vaccination.

In **Chapter 4**, we investigated the safety and immunogenicity of yellow fever vaccination in the context of drug-induced immune suppression. Live vaccines have an inherent risk of uncontrolled growth of the attenuated vaccine strain in the immunocompromised host (5). In case of the yellow fever vaccine, this may result in the potentially fatal yellow fever associated neurotropic and viscerotropic disease (respectively YEL-AND and YEL-AVD). Over the years, several immunocompromised patients have received a yellow fever vaccine despite their immunosuppressed state. In **Chapter 4** we determined the durability of the antibody response in this group with two different serological assays.

In 2013 the World Health Organization (WHO) revised the evidence and recommendations for yellow fever vaccine (19). Following the advice of the Strategic Advisory Group of Experts on Immunization (SAGE) Yellow Fever Working Group (20), the WHO position was that "a single dose of YF vaccine is sufficient to confer sustained life-long protective immunity against YF disease; a booster dose is not necessary". However, the WHO stated that additional research was needed for special risk groups, such as infants, pregnant women, the immunosuppressed, and people aged 60 and over. In **Chapter 5** we explore the duration of protection in more detail by means of a retrospective follow-up of neutralizing antibody titers in 99 healthy vaccinees that were vaccinated up to 40 years prior, and also by comparing functionally competent yellow fever specific $CD8^+$ T cells between recently vaccinated volunteers and a subset of the 99 volunteers.

In **Chapter 6**, we determined the antibody response to the whole cell/B subunit oral cholera vaccine (WC-BS) in kidney transplant recipients on different immunosuppressive regimes. In 2008 a consensus meeting of experts suggested that WC-BS might be considered to prevent Enterotoxic E. coli -related traveller's diarrhea, especially in travellers at risk to develop serious illness (21). This recommendation was based on the antigenic similarity of the toxin B subunit of Vibrio cholera and

E. coli (22). Although this notion was subsequently not supported by evidence (23), vaccine responses can still be used to gauge the relative immunosuppressive effect of different immunosuppressive regimes on primary mucosal immune response in transplant patients as cholera toxin B subunit is a neo-antigen for most Western transplant recipients. This may help clinicians decide whether protection from vaccination is attainable in their patients.

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Chapter 2

Single visit rabies pre-exposure priming induces a robust anamnestic antibody response after simulated post-exposure vaccination: results of a dose-finding study

J Travel Med. 2017 Sep 1;24(5).

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2.1 Abstract

Background

The current standard 3-dose intramuscular rabies PrEP schedule suffers from a number of disadvantages that severely limit accessibility and availability. The cost of is often prohibitive, it requires 3 visits to the clinic, and there are regular vaccine shortages.

There is accumulating evidence that PrEP can be shortened to 2 visits without affecting seroconversion rates or memory formation. The primary objective of this dose finding study is to determine the optimal pre-exposure priming regimen that would require only a single visit to the clinic in order to produce an adequate memory response in all subjects one year later.

Methods

Volunteers (N=30) were randomly assigned to 4 study arms: 1 standard dose intramuscular (IM) dose of PVRV (purified Vero cell rabies vaccine, Verorab), and 1/5th, 2/5th or 3/5th- fractional intradermal (ID) dose of PVRV in a single visit. All subjects received a simulated rabies post-exposure prophylaxis (D0, D3) one year later. Rabies virus neutralizing antibodies (RVNA) were determined by virus neutralization microtest (FAVN) on D0, D7, D28, Y1, and Y1+D7.

Results

28 out of 30 subjects (93%) seroconverted 1 month after primary vaccination; 1 subject in the 1-dose IM arm and 1 in the 1/5th-fractional dose ID arm did not. After 1 year, 22 out of 30 subjects (73%) no longer had RVNA above 0.5 IU/mL, with no discernible difference between study groups. After 1 year, all 30 subjects mounted a booster response within 7 days after simulated PEP, with the highest titers found in the single dose IM group ($p<0.03$).

Conclusions

This dose finding study demonstrates that priming with a single dose of rabies vaccine was sufficient to induce an adequate anamnestic antibody response to rabies PEP in all subjects one year later, even in those in whom the RVNA threshold of 0.5 IU/mL was not reached after priming.

2.2 Introduction

Rabies is a fatal viral encephalitis that can infect all mammals. Ninety-nine percent of human cases result from dog bites. According to the most recent global estimates canine rabies causes 59,000 deaths in humans annually[1]. The vast majority occur in Asia (59.6%%) and Africa (36.4%), and are related to insufficient coverage of dog vaccination and absence of rabies post-exposure prophylaxis (PEP)[2]. For example in Indonesia, areas previously free of rabies are seeing a catastrophic re-emergence after initially successful elimination programs failed [3, 4] due to incomplete canine vaccination coverage[5].

The total cost of rabies has been estimated to be between 9 and 124 billion dollars per year[1, 6]. This cost can be mitigated in several ways, amongst which are reducing rabies disease prevalence through combating animal rabies, and immunization of vulnerable human populations. Although canine vaccination is the most cost-effective measure, this is still inadequately pursued [2] and the best option for the short to medium term is to improve availability and affordability of human rabies vaccination[1].

After a bite, scratch or lick of a rabid animal, rabies virus probably multiplies in the muscles surrounding the exposed site[7]. After a variable period of several days to even years, the virus enters the nervous system. This period is the only window of opportunity to stop disease and save the bite victim's life. Rabies virus can be stopped by thorough wound cleansing with soapy water, followed by wound disinfection with an iodine antiseptic solution and PEP[8]: the administration of a series of 4 or 5 doses of modern cell-derived rabies vaccine, combined with perilesional anti-rabies immunoglobulin (RIG) if necessary. Over 20 million people receive rabies PEP each year [9], mostly in resource-poor countries.

If the bite victim was vaccinated against rabies before the exposure occurred, revaccination with just 2 doses will suffice to boost a rapid and robust memory response. This memory response eliminates the need for RIG. It is the most important purpose of rabies pre-exposure prophylactic vaccination (PrEP): to build an immunological memory that provides a rapid and adequate anamnestic antibody response upon revaccination. PrEP vaccination also induces a transient antibody titer that may protect against (unnoticed) exposure.

The current standard 3-dose intramuscular PrEP schedule suffers from a number of disadvantages that severely limit accessibility and availability. The cost of a full PrEP course is often prohibitive, it requires 3 visits to the clinic, and there are regular vaccine shortages. Travelers in particular often don't have enough time to complete the PrEP series between their travel clinic visit and date of departure. This is becoming increasingly relevant as the number of PEP consultations is increasing year-on-year and the majority of those requiring PEP did not receive PrEP [10].

The administration of a fractional dose through the intradermal (ID) route in the standard schedule of 3 visits in 3 weeks may reduce costs significantly [11]. In addition, several studies indicate that PrEP can be shortened to 2 visits by using multi-site ID injection of the vaccine [12]. Such dose-sparing regimens may prove to be an excellent way to increase accessibility and availability of rabies PrEP [13]. There are indications that even one clinic visit using multi-site ID injection results in sufficient seroconversion: two ID injections of PCECV on day 0 resulted in 71-77% seroconversion rate at day 35 [14] and four ID injections of HDCV on day 0 resulted in 100% seroconversion rate at day 14[15]. A single intramuscular dose resulted in 100% seroconversion in 18 subjects 35 days after HDCV[16], and in 97% seroconversion in 33 subjects 35 days after PCECV[14]. The subjects in these studies demonstrated a memory response after a booster dose.

From these studies we hypothesize that a single rabies vaccine dose is sufficient to prime the immune system in such a way that it will result in a fast memory response after revaccination, even in the absence of seroconversion after primary vaccination. The primary objective of this dose finding study therefore is to determine the optimal pre-exposure vaccination regimen that would require only a single visit to the clinic in order to produce an adequate memory response in all subjects after one year.

2.3 Methods

Study population

Volunteers were recruited through advertisements in Leiden University buildings. An incremental incentive was provided to all subjects who completed the entire study protocol. Volunteers between 18 and 65 years old were included if they were in good health, willing and able to adhere to the study regimen, and able to provide informed consent. Exclusion criteria were any previous rabies vaccination, known or suspected allergy against vaccine components, history of serious adverse

reactions after vaccination, history of syncope due to needle sticks, immunocompromized state either through medication or medical condition, receiving blood products in the last 3 months, hydroxychloroquine or mefloquine use, history of any neurological disorder, use of anticoagulants, breastfeeding, a positive urine pregnancy test, refusal to use contraceptives during the study period, high grade fever, acute infectious disease other than seasonal cold, and participation in another trial in the last 3 months.

Study design

This is a dose-finding study performed according to a non-blinded comparative randomized clinical trial design. The study was performed at the Travel Clinic of the Department of Infectious Diseases at Leiden University Medical Center (LUMC, Leiden, The Netherlands) from November 2014 until March 2016. Subjects were randomly assigned to 4 regimens for primary rabies vaccination using computer-generated permuted block randomization:

- A: 1-site 0.5 mL intramuscularly (IM) (standard dose)
- B: 1-site 0.1 mL ID (equivalent to 20% of standard dose)
- C: 2-site 0.1 mL ID (40% of standard dose)
- D: 3-site 0.1 mL ID (60% of standard dose).

All injections were given at the same visit on a single day. Multi-site injections were given at distinct body sites in order to address the maximum number of lymph node stations: the deltoid region for the 1- and 2-site regimens, and for the 3-site regimen also the quadriceps region. After 1 year, all subjects received 2 standard doses in the ipsilateral deltoid muscle on day 0 and day 3 to simulate rabies post-exposure prophylaxis (figure 1).

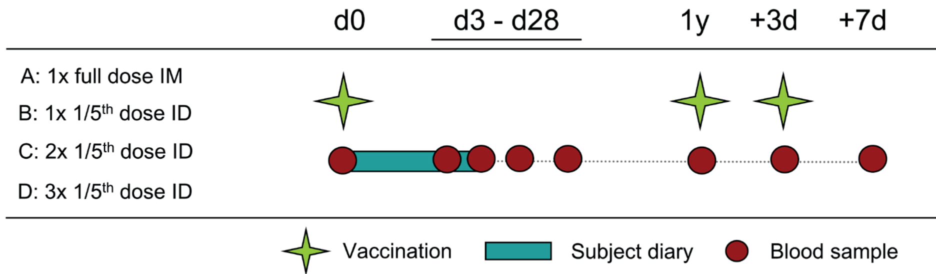


Figure 1: Subject timeline and study logistics. The 4 study arms presented on the left followed an identical course starting with the experimental single-visit vaccination at day 0, followed 1 year later by the standard intramuscular post-exposure vaccination schedule.

Vaccination, blood sampling and adverse events.

The vaccine used was a purified Vero cell rabies vaccine (PVRV, Verorab®, Sanofi Pasteur MSD). Verorab® in the original formulation contains per 0.5 mL dose ≥2.5 IU lyophilized inactivated rabies virus (strain PM/WI 38-1503-3M). All primary vaccinations were performed with the same vaccine batch (lot no. K1382-1, exp. date 6/2016; potency 3.2 IU/dose as determined by the manufacturer). The lowest priming dose used was 0.6 IU (group B; 1x 0.1 mL ID).Simulated PEP vaccinations were performed with different batches (lot no. 1126-3, exp. 3/2017 and L1446-1 exp. 8/2017). Between 2013 and 2016, Verorab® was the only rabies vaccine available in the Netherlands.

After each intradermal injection, wheal size and amount of leakage was measured. Wheal size was quantified as wheal diameter across 2 perpendicular axes, the average of which was taken as single value wheal size.

Subjects were asked to complete a diary for 5 days after primary vaccination. The following adverse events were solicited in the diary: local tenderness, swelling, itching, myalgia, erythema (grouped together in the analysis as 'local reactogenicity'), headache, fatigue, medication taken and extent to which symptoms influenced day-to-day functioning. After PEP vaccination, these events were solicited orally.

Venapunctures were performed on days 0, (2-)3, (6-)7, 14(-16), and 28(-35). After 1 year, venapunctures were performed at baseline (day 0), before the second booster dose (day 3) and at day 7. Blood samples were processed on the same day and serum was stored at -80 °C until analysis.

Fluorescent Antibody Virus Neutralization (FAVN)

The FAVN is a virus neutralization microtest adapted from the rapid fluorescent focus inhibition test (RFFIT) by Cliquet et al[17]. Both the FAVN and the RFFIT are recognized by WHO and OIE (World Organization for Animal Health) as the gold standard for rabies serology. As reference serum, the OIE dog serum calibrated against WHO's 1994 human reference serum was used (2nd international reference serum) [18]. The actual antibody level necessary for protection is unknown, but a level above the validated cut-off of 0.5IE/mL is definitive proof of seroconversion [18]. For this trial, the FAVN was performed in the high containment unit (HCU) of the Central Veterinary Institute (CVI), Wageningen University and Research Center (WUR), Lelystad, The Netherlands.

In brief, sera to be tested were complement inactivated for 30 minutes at 56 °C. Serial 3-fold dilutions from 1:3 to 1:81 of controls and test sera were mixed with 100 TCID50 rabies virus (CVS-11 strain/ ATCC VR959, ANSES, Nancy, France) and assayed in quadruplicate. The mixture was incubated for 1 hour at 37 °C and transferred to 96-well plates (Greiner Bio One BV, Alphen aan den Rijn, The Netherlands). BHK-21 cells in monolayer culture were trypsinized, suspended in DMEM + Glutamax (Invitrogen, subsidiary of Thermo Fisher Scientific, Waltham, Massachusetts, USA) and added to the virus-serum mixtures. The 96-well plates were then incubated for 2 days at 37 °C in 5% CO2. After incubation, the plates were fixed with 80% acetone, air-dried and stained with fluorescein isothiocyanate conjugated (FITC) anti-rabies serum (Fujirebo Diagnostics, Philadelphia, USA).

Fluorescent wells were counted quantitatively under an inverted fluorescence microscope. The decimal log dilution at which 50% of wells were neutralized (logD50) was calculated according to the Spearman-Kärber method and the titer reported in international units according to the following formula: ((10^logD50 of tested sample)/10^(logD50 of OIE reference))*0.5.

Sample size and analysis

This was a dose-finding trial. As such no formal sample size calculation was performed. In consultation with the medical statistician, it was decided to include 5 subjects per arm and an additional 5 per arm in the two arms with the lowest seroconversion rates one month after primary vaccination. The lowest seroconverting arms were chosen for expansion in order to facilitate demonstration of the primary hypothesis: 100% booster response after 1 year even in the absence of seroconversion after primary vaccination. A booster response was defined as seroconversion within 7 days post booster,

or a 4-fold increase of RVNA titer in those groups with a pre-booster RVNA >0.5 IU/mL.

Participant demographics and adverse events were analyzed using descriptive statistics. Reverse cumulative distributions were compared using the 2-sample Kolmogorov-Smirnov Z-test. Geometric titers and fold increase were analyzed using students t-test. Correlations were analyzed visually using scatter plots. Analyses were performed using SPSS Statistics version 23 (IBM Corp., Armonk, New York, USA), Excel version 2010 (Microsoft Corp., Redmond, Washington, USA) and Prism version 6 (GraphPad Software, La Jolla, California, USA).

Ethical considerations

The study was approved by the local Medical Ethics Committee (METC) of the Leiden University Medical Center (LUMC, Leiden, the Netherlands) and registered in clinicaltrials.gov under NCT02276625. All participants provided informed consent before enrollment.

2.4 Results

In total, 30 subjects were enrolled, all of whom completed the study including the simulated PEP one year after primary vaccination. Subjects were between 18 and 31 years of age (median 21.9) and were predominantly female (70%) (table 1).

One month after priming (experimental primary vaccination), there was an overall 93% (95% CI 84-100%) seroconversion rate for the one-visit priming schedules. Nine out of 10 subjects seroconverted in arm A (1-IM) and 9 out of 10 in arm B (1/5th-ID). Five out of 5 subjects seroconverted in arm C (2/5th-ID) and 5 out of 5 in arm D (3/5th-ID) (table 2). One month after priming, the geometric mean titers (GMT) were not different between groups, and there was no dose-response relationship with regards to antigen dose at priming (table 2; figure 2).

Table 1: Participant demographics, group size and comparative characteristics at baseline

	A 1 dose IM	B 1/5th dose ID	C 2x 1/5th ID	D 3x 1/5th ID
No of subjects	10	10	5	5
Age (yrs, [median, range])	21.5 (20-31)	20.5 (19-25)	23 (18-28)	22 (19-25)
BMI (mean, range)	23.9 (21-34)	23.4 (21-27)	25.2 (20-38)	22.3 (21-24)
Sex (# female)	8/10	8/10	2/5	3/5
Vaccine priming dose (IU)	3.2	0.6	1.2	1.8

Table 2: Serology, wheal size and side effects.

	A 1 dose IM	B 1/5th dose ID	C 2x 1/5th ID	D 3x 1/5th ID
Interval primary serology (mean days)	29.1	29.5	29.8	28.6
Interval primary-booster (mean days)	372	369	368	369
Average wheal diameter (mm)	NA	8.5	8.7	10.0
Serology				
GMT at baseline (IU/mL)	0.0 [0.0-0.0]	0.0 [0.0-0.0]	0.0 [0.0-0.0]	0.0 [0.0-0.0]
GMT at 7 days post primary (IU/mL, [95% CI])	0.0 [0.0-0.0]	0.0 [0.0-0.0]	0.0 [0.0-0.0]	0.0 [0.0-0.0]
RVNA range (IU/mL)	0.0-0.1	0.0-0.1	0.0-0.1	0.0-0.1
GMT 1 month post primary (IU/mL, [95% CI])	2.2 [1.3-3.9]	2.0 [1.1-3.8]	6.7 [2.9-15.4]	4.2 [1.4-13.0]
RVNA range (IU/mL)	0.5-11.6	0.4-7.4	2.5-29	0.8-16.8
Seroconversion, no. of subjects	9/10	9/10	5/5	5/5
GMT pre-booster baseline (IU/mL, [95% CI])	0.4 [0.2-0.6]	0.0 [0.0-2.0]	0.2 [0.1-0.4]	0.5 [0.3-0.8]
RVNA range (IU/mL)	0.1-1.3	0.0-1.3	0.1-0.6	0.2-1.0
Seroconversion, no. of subjects	3/10	2/10	1/5	2/5
GMT 3 days post booster (IU/mL, [95% CI])	0.3 [0.2-0.6]	0.0 [0.0-1.5]	0.3 [0.1-0.6]	0.5 [0.9-0.3]
RVNA range (IU/mL)	0.1-1.3	0.0-1.3	0.1-0.7	0.2-1.3
GMT 7 days post booster (IU/mL, [95% CI])	63.9 [45.1-90.6]	22.6* [10.8-47.0]	13.0* [7.7-22.0]	20.1* [12.9-31.5]
Fold increase in GMT vs pre-booster [95% CI]	252 [114-390]	86 [28-144]	67* [38-96]	48* [26-70]
RVNA range (IU/mL)	26.6-239.2	3.0-239.2	5.1-26.6	11.7-34.8
Seroconversion, no. of subjects	10/10	10/10	5/5	5/5
Side effects and AEs after primary vacccination				
Myalgia	3/10	2/10	2/5	1/5
Localized erythema	0/10	4/10	3/5	4/5
Headache	3/10	3/10	2/5	0/5
Fatigue	3/10	1/10	1/10	1/10

* significant difference compared to arm A, students t-test

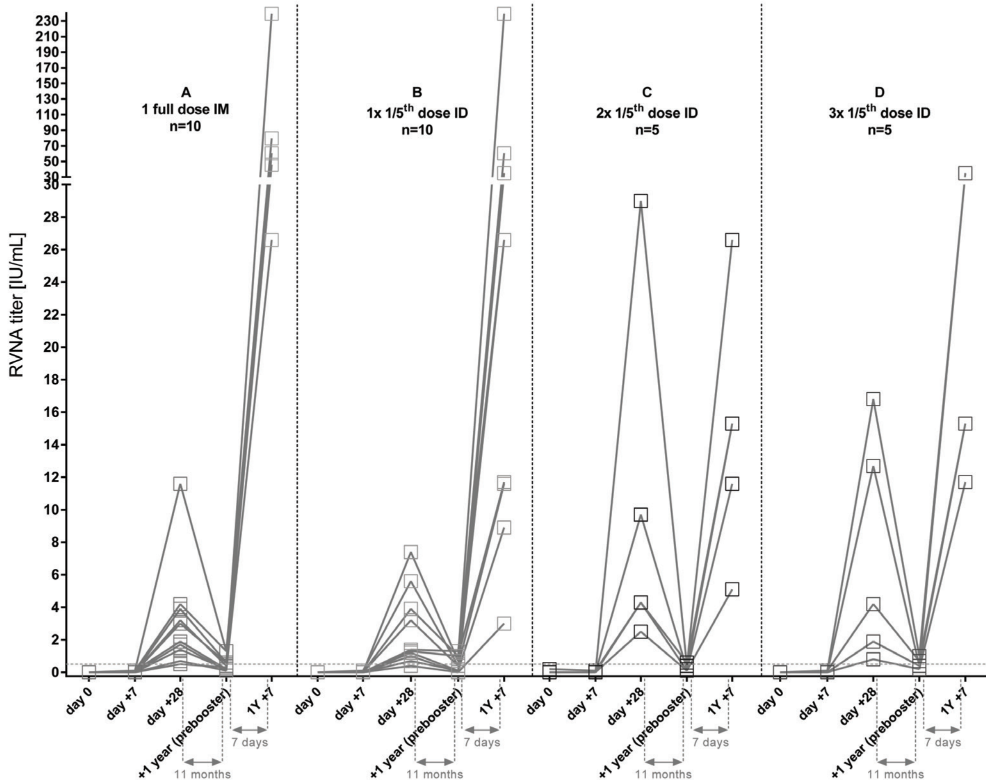


Figure 2: Individual titers plots. Individual titer plots showing per subject RVNA titers at baseline, day 7 and day 28 after primary vaccination, titers after 1 year (prebooster) and 7 days post booster. Note the lack of response 7 days post primary as opposed to 7 days post booster and the high post-booster titers in the group primed with 1 intramuscular injection.

One year after primary vaccination, 8 out of 30 subjects still had RVNA titer >0.5 IU/mL before simulated PEP. The distribution was as follows: 3 out of 10 in arm A; 2 out of 10 in arm B; 1 out of 5 in arm C, and 2 out of 5 in arm D (table 2).

All 30 subjects seroconverted within 1 week of the first booster dose (table 2; figure 2), even those who did not seroconvert after primary vaccination. Therefore, all experimental study arms satisfied the primary endpoint. At day 7 after revaccination GMT increased 251-fold in arm A, and between 48- to 86-fold in the intradermal arms (table 2). The difference in fold increase was significant ($p<0.03$) for arm A compared to arms C and D. Although the 1-IM arm (A) showed the highest GMT post-booster, no dose-response relationship was found in our study either when all groups were compared nor in the ID arms separately. Serology performed at day 3 post-booster did not show a difference in GMT

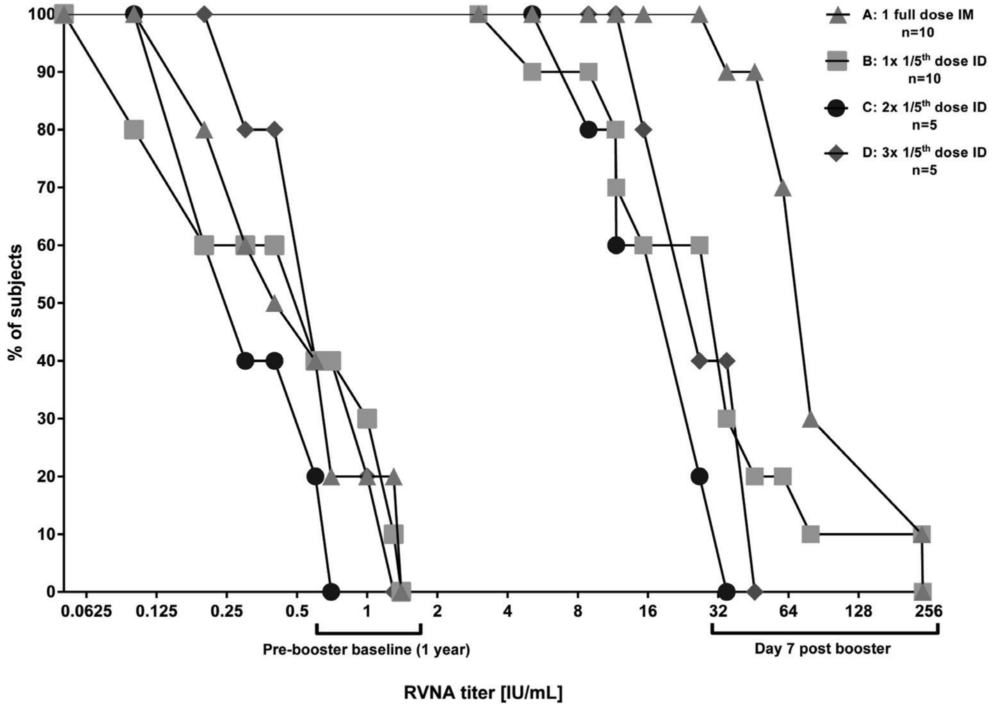


Figure 3: Reverse cumulative distribution of RVNA antibody titers before and after booster vaccination. Reverse cumulative distribution curves of RVNA titers per group, shown for the pre-booster baseline at 1 year (on the left) and 7 days after the post-exposure boosters (on the right). Note the highest post-booster titers in the group primed with 1 intramuscular injection ($p<0.015$, 2-sample Kolmogorov-Smirnov Z-test).

from the pre-booster baseline.

Reverse cumulative distribution curves of RVNA titers at day 7 post booster showed the highest titers in the 1-IM group (figure 3, $p<0.015$ for each intradermal group versus intramuscular, Kolmogorov-Smirnov Z-test), while no difference between the intradermal groups was found. There was no correlation between local reactogenicity and RVNA titer at day 28 post primary or day 7 post booster (data not shown). The average wheal diameter after intradermal injection was 9.2 mm (table 2).

There were no serious adverse events (SAE). Adverse events occurred after both the primary and booster vaccinations (table 2). After the primary vaccination, 12 out of 30 subjects did not report any side effect. The remaining subjects reported localized erythema (only ID groups, 11/30), myalgia (8/30), headache (8/30), and fatigue (6/30). In the intradermal groups, a little over half of subjects experienced painful erythema of around 5 mm at the site of injection for the first few days.

In some instances, a small red spot remained until 4 weeks after injection, predominantly over the quadriceps area. After 1 year, no evidence of intradermal injection could be found on the skin of any of the subjects. After booster vaccination, myalgia occurred in 8/30 subjects with one subject also reporting swollen axillary lymph nodes and another reporting fatigue.

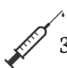
Two subjects finished the study outside the pre-defined time limits; one subject 4 months early and one 4 months late, both in arm A (1-IM). Removing their data from analysis did not change the results (data not shown).

2.5 Discussion

This dose finding study demonstrates that priming with a single dose of rabies vaccine was sufficient to induce an adequate anamnestic antibody response to rabies PEP one year later. A robust memory response was seen in all subjects across all experimental priming regimens, regardless of dose or route of administration, even in those in whom the RVNA threshold of 0.5 IU/mL was not reached after priming.

Although most subjects seroconverted after primary vaccination, it was short-lived, as RVNA titers dropped significantly during the first year. Only 27 percent of subjects had a titer >0.5 IU/mL after one year. The rapid drop in RVNA titers is most likely explained by the lower number of effector cells that are formed after a single injection, in comparison to the standard 3-dose PrEP where further expansion of the pool of effector cells is expected to occur after the second and third dose [19]. After standard 3-dose PrEP vaccination, RVNA persist beyond the first year[20-22], and only start to drop below 85% after the second year[23]. Therefore, repeated vaccinations over several weeks or months are needed if prolonged persistence of RVNA after vaccination is required[24, 25]. Single dose primary vaccination however did induce adequate numbers of memory cells given the robust memory response that was seen after booster vaccination.

A booster response is characterized by a rapid and strong anamnestic antibody response after revaccination. Although straightforward in principle, the quantitative definitions of ‘rapid’ and ‘strong’ used in literature are quite diverse. We chose seven days after revaccination to differentiate a secondary or booster response from a primary antibody response, which generally occurs between 14 and 28 days after vaccination [19]. A significant rise in antibody titres is classically defined as seroconversion or an increase in antibody titres of more than two dilution steps, to account for the



inter-assay variability of serological tests. In this study all subjects had at least a 10-fold increase in RVNA titres at day 7 after revaccination. In contrast, no serological response was detectable in any of the subjects 7 days after the initial priming dose.

Our study has several strengths including the randomized controlled design, use of gold standard serology, blinding of laboratory personnel who performed the serology, and no loss to follow-up. Limitations include the small sample size and the fact that only young healthy adults were included. This may impact external validity, as it is known that the elderly have lower seroconversion rates after rabies vaccination [26]. In addition, it is unknown how long immunologic memory persists beyond one year. Lastly, standard 3-dose PrEP vaccination was not included for comparison. In future studies, a control arm with the current standard schedule should be included.

Intradermal administration of vaccines is thought to enhance immunogenicity because of the high density of antigen presenting cells (APC) in the papillary dermis [27]. Furthermore, the papillary dermis facilitates rapid trafficking of antigen and activated APC to draining lymph nodes where subsequent T-cell and B-cell activation and initiation of an adaptive immune response can occur [28] [29]. As a consequence, intradermal vaccination allows for the use of less vaccine than intramuscular or subcutaneous administration to obtain similar antibody responses, saving costs and increasing availability of vaccine in resource-poor regions of the world [30].

The feasibility of an abbreviated schedule of intradermal rabies vaccination was first demonstrated by Turner et al in 1976[31], later followed by Warrell and others[12, 32-34]. Turner also demonstrated that a single intradermal injection of 0.1 mL HDCV could prime the immune system leading to 100% seroconversion 28 days after a single intramuscular booster six months later [31, 35]. Although it was not the primary objective of their study, Brinkman et al found that a single intramuscular injection of HDCV resulted in 100% booster response within 7 days following a single intramuscular booster three months later[16]. Our study extends these findings, demonstrating that priming with a single intradermal fractional dose or intramuscular standard dose of a modern Vero cell-based rabies vaccine results in a robust memory response in all subjects, one year later.

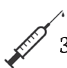
We specifically chose to include the intramuscular route in this study as well, because it is technically less demanding than intradermal injection. In addition, in many countries the intramuscular route is the only licensed route of administration of rabies vaccine. Although individual RVNA titers varied substantially, we found that priming by the intramuscular route with a standard dose of rabies

vaccine resulted in a significantly higher fold-increase of post-booster GMT compared to priming by the intradermal route with a fractional dose. RVNA titers achieved post booster in this study were similar to those found after boosting subjects that received a standard pre-exposure vaccination [36].

If antibody responses in secondary immune responses are related to the number of memory cells, it would be reasonable to conclude that more memory cells were formed in the intramuscular group. Whether this is explained by the higher vaccine dose or by the intramuscular route remains to be determined. We did not observe a dose-response relationship between post-booster GMT and the fractional dose of rabies vaccine used for priming by the intradermal route. Possibly, this is because of the small sample size. Tauber et al. did find a dose-response relationship after single visit multi-fractional dose ID vaccination despite similar small numbers of subjects [34]. Beran et al also found a clear and significant linear correlation between vaccine dilution and resultant GMT, both early and late after intradermal vaccination with a single lot of purified chick embryo cell rabies vaccine (PCECV) according to the Thai Red Cross post-exposure regimen[37].

If confirmed, our findings may have a profound impact on preventive rabies vaccination strategy, especially in travelers. Although bite wounds occur at a rate of 1 in 300 travellers per month of stay [27, 29, 38], most travellers do not receive standard 3-dose pre-travel PrEP because of high costs and insufficient time between visit to the travel clinic and departure. On the other hand, travelers only run a risk of rabies exposure over a limited period of time. If preventive rabies pre-exposure priming with a single dose of rabies vaccine would suffice to cover this period, PrEP rabies vaccination would come within reach of many more travellers. This simplified schedule could be repeated for future travels. After three vaccine doses a lifelong rapid and adequate anamnestic antibody response after revaccination can be expected as with the standard 3-dose PrEP vaccination schedule. It is important to stress that vaccinated travelers must seek immediate medical attention after a bite accident. In a recent case series on rabies PEP it was found that 50 % of the travelers had received their first injection of rabies vaccine in the destination country within 24 hours [10] and 60% within 48 hours [personal communication with Wieten RW].

Before implementation, we would like to summarize the way forward as follows. First we need to demonstrate that, within a specified time window, the anamnestic RVNA response to simulated rabies PEP after single-dose priming is non-inferior to standard 3-dose PrEP. Secondly, we have to establish for which age groups single-dose priming would provide RVNA titres >0.5 IU/mL for a



sufficient time period (for example 3 or 6 months). Finally, we need to determine how the financial and logistical advantages of the single-dose priming relate to those of the standard 3-dose PrEP in specific risk groups.

In conclusion, effective rabies pre-exposure vaccination for travelers may be achieved in a single visit using a modern vaccine, with 100% booster response after 1 year even in those who do not seroconvert after the priming dose. Adequately powered non-inferiority studies should follow up on the results from this dose finding study and should include the standard intramuscular PrEP schedule as a control arm.

Author contributions

LV conceived of the research idea; EJ researched, designed and executed the trial under supervision of LV; EJ and LV analyzed the data and wrote the manuscript.

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Disclosure

The authors have declared no conflicts of interest. No writing assistance was used in the preparation of this manuscript.

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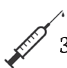

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Chapter 3

Safety and immunogenicity of fractional dose intradermal injection of two quadrivalent conjugated meningococcal vaccines

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3.1 Abstract

Background

Vaccination with conjugated meningococcal vaccines is the best way to prevent invasive meningococcal disease. Changes in serogroup epidemiology have led to the inclusion of quadrivalent vaccines in the national immunization programs of several countries, but vaccines are frequently in short supply. Intradermal administration has the potential to increase vaccine availability through dose reduction, without sacrificing efficacy. It has never before been investigated for glycoconjugate meningococcal vaccines.

Methods

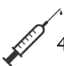
Different fractional doses of two quadrivalent meningococcal conjugate vaccines (MenACWY-CRM197 (Menveo®) and MenACWY-TT (Nimenrix®)) were administered intradermally to sequential groups of 4 participants, according to an adaptive dose escalation design, starting at 1/10th of the original dose. Booster doses were given after 4-6 months based on interim serology results using a multiplex bead-based assay (MIA). Final analyses were based on serum bactericidal antibody titers (rSBA).

Results

A total of 12 subjects were enrolled (average 25 years old, range 19-48). MenACWY-CRM197 became unavailable during the course of the study and was only evaluated for a 1/10th dose. This dose resulted in less than complete seroprotection for serogroup A but complete protection against the other serogroups. MenACWY-TT was evaluated for a 1/10th and 1/5th dose level. Both fractional doses of MenACWY-TT resulted in complete seroprotection against all vaccine serogroups. Geometric mean titers 1 month after vaccination were lower and decayed faster in the MenACWY-CRM197 group. Adverse events were mild and there were no serious adverse events.

Conclusion

Fractional intradermal vaccination against meningococcal disease with quadrivalent conjugate vaccine appears to be safe and effective in our small dose finding study. Tetanus toxoid conjugated vaccine (Nimenrix®) shows a trend towards higher antibody levels compared to CRM197-conjugated vaccine (Menveo®). The 1/5th fractional dose of MenACWY-TT appears to result in higher antibody levels than does the 1/10th dose. These results can be used for a larger non-inferiority study.



3.2 Introduction

Neisseria meningitidis is a major cause of invasive bacterial infections globally. Virulent strains of N. meningitidis have a polysaccharide capsule, which is the major virulence factor for this bacterium. There are 13 diverse polysaccharide capsules, but only A, B, C, W, X and Y commonly cause invasive infections. The polysaccharide capsule of N. meningitidis induces a protective antibody response and there is an inverse correlation between the incidence of invasive meningococcal disease and the age-related acquisition of serum bactericidal antibodies [1]. Vaccination is generally accepted as the best way to prevent invasive meningococcal disease caused by serotypes A, C, Y, and W [2]. In recent years, 2 quadrivalent conjugate vaccines (Menveo®, MenACWY-CRM197 and Nimenrix®, MenACWY-TT) have been registered in Europe to replace the unconjugated (quadrivalent) polysaccharide vaccine.

3

Although serogroup A was a common cause of invasive meningococcal disease (IMD) in Europe up to the 1950s, it has now disappeared [3, 4]. In 2002, after an outbreak of IMD caused by serogroup C, MenC-TT was added to the Dutch National Immunization Program after a mass vaccination campaign with a coverage of 94%. The decline in serogroup C disease is mainly attributable to the use of conjugated meningococcal group C vaccines [5]. In recent years, the rise of serogroup W from South America to the UK and now to the rest of Europe has been the most notable change in European meningococcal epidemiology [6]. This had led several countries to include or consider including a quadrivalent meningococcal vaccine into their national immunization program to replace the meningococcal C vaccines. Recently, the UK and Australia have included a quadrivalent meningococcal vaccine and the UK was the first country to include meningococcal B vaccine (Bexsero®) in the national immunization program[7].

Normally, vaccines are administered into the muscle (intramuscular administration, IM). However, the skin (dermis) contains a much higher density of antigen presenting dendritic cells than does muscle [8]. The skin lymphatic system is extensively organized into several plexus systems, which aids efficient transport of antigen presenting dendritic cells to the regional lymph nodes [9]. As a consequence, a lower vaccine dose introduced directly into the dermis (intradermal administration, ID) might be sufficient to achieve a protective immune response. This principle has already been demonstrated for rabies, yellow fever, inactivated polio and seasonal influenza vaccine[10].

In this dose-finding trial we investigated the safety and immunogenicity of intradermal

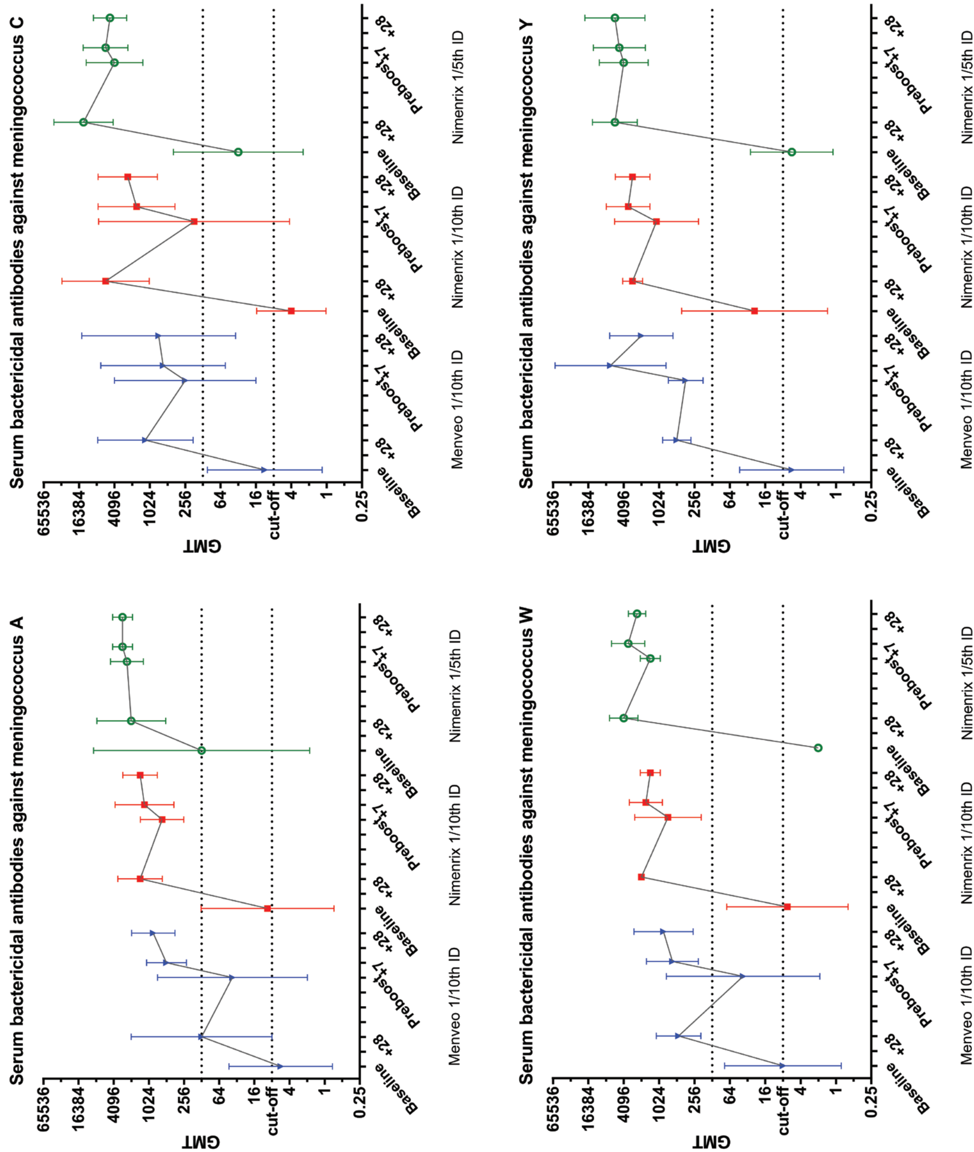


Figure 1: Geometric mean titres (GMT) per serogroup, subdivided per trial arm. The 3 trial arms are displayed within each graph. The time points for each trial arm are connected for clarity, starting from baseline (inclusion) and ending at 28 days post booster. Cut-offs are indicated by dotted lines at 1:8 and again at 1:128. Please note the decaying antibody levels prior to the booster vaccinations.

administration of fractional dose MenACWY-CRM197 and MenACWY-TT. It is the first study of intradermal use of a conjugated vaccine.

3.3 Methods

Study design

Open-label uncontrolled randomized intervention study. The study was performed with 2 vaccines: MenACWY-CRM197 (Menveo®) and MenACWY-TT (Nimenrix®). An adaptive dose escalation rule based on interim analyses of safety and immunogenicity was used to increase the fractional vaccine dosage as the study progressed. A minimum of 8 and a maximum of 16 subjects per vaccine could be included, based on discussions with experts on dose escalation studies. Inclusion started at the fractional 1/10th dose level and dose escalation was limited to a 1/5th fractional dose. Subjects were randomly assigned to dose level and vaccine in groups of 4 subjects each. All subjects followed the same schedule: primary vaccination with a fractional dose and blood sampling on day 0 followed by blood sampling on day 27-29, and after 4-6 months on days 0 and 7 and 27-29 after revaccination. No IM control arm was included because the study was not intended to compare ID administration to IM; the goal was to establish an immunogenic dose that could be assessed against IM administration in a subsequent much larger comparative trial.

Due to insufficient immunogenicity after the highest dose (1/5th fractional dose) according to the interim serological analysis using a multiplex immunoassay (MIA), the study was amended and a booster dose of a single intradermal injection was added with the same fractional dose as previously received by the subject. Because MenACWY-CRM197 (Menveo®) became unavailable in the Netherlands during the study, this vaccine was only evaluated in the primary 1/10th dose level. All subjects, including those who initially received MenACWY-CRM197 (Menveo®), were boostered with MenACWY-TT (Nimenrix®).

Vaccination

Two quadrivalent conjugated meningococcal vaccines were used: MenACWY-CRM197 (Menveo®, GSK, lot no. M11026) and MenACWY-TT (Nimenrix®, Pfizer, lot no. A90CA001E). Both vaccines consist of capsular oligosaccharides of 4 meningococcal serogroups conjugated to a bacterial carrier protein.

For the 1/5th fractional dose, 0.1 mL of the original vaccine formulation was injected intradermally. Because the quality measures for intradermal vaccination are standardized on a 0.1 mL injectable volume, the 1/10th fractional dose was also administered in a 0.1 mL volume (achieved by adding twice the amount of diluent). The vaccine doses were injected into the skin on the dorsal side of the forearm using disposable Beckton Dickinson U-100 Micro-Fine™ insulin syringes with integrated 29G needle.

Vaccine safety

Adverse events were solicited for 7 days after primary and booster vaccination using a paper diary provided to each subject. Adverse events were also checked by telephone interview 7 days after the primary vaccination. Furthermore, an independent data and safety monitoring board (DMSB) was established, because this study involved the first trial of intradermal injection of conjugated polysaccharide vaccine. Due to very limited local reactogenicity after primary vaccination, even in those subjects who were seroprotected against MenC at inclusion, the DSMB was discontinued for the booster doses.

Study population and inclusion criteria

Subjects were recruited through advertisements in Leiden University buildings. Volunteers between 18 and 65 years old were included if they were in good health, willing and able to adhere to the study regimen and provide informed consent. Exclusion criteria were a known previous quadrivalent meningococcal vaccination, previous meningococcal infection, allergy to any of the vaccine components, close contact with a person known to be Neisseria positive within the last 60 days, (family) history of Guillain-Barré Syndrome, known or suspected immune deficiency either congenital or acquired, administration of blood products in the last 3 months, use of anticoagulants, pregnancy, refusal to use contraceptives during the study period, fever, acute infectious disease other than seasonal cold, and participation as a subject in another trial in the last 3 months. An incentive of 45 euros was provided to all subjects who completed the primary vaccination and a further 45 euro after the booster phase.

Immunogenicity analysis

Serology was performed at the National Institute of Public Health and the Environment (RIVM, Bilthoven, the Netherlands). The immunogenicity and antibody persistence against MenACWY

polysaccharides were initially assessed by fluorescent-bead-based multiplex immunoassay (MIA) during the study, as previously described [11, 12].

Gold standard serology was performed at the end of the study by measuring the level of specific functional antibodies for each serogroup using an in-house serum bactericidal antibody assay (SBA) with baby rabbit complement (Pelfreez, ref#360160) and MenA, MenC, MenW and MenY strains 3125, C11, MP01240070, S-1975, respectively. The bactericidal titer was defined as the dilution of the test serum that resulted in ≥50% killing after 60 minutes incubation with a titer of ≥8 as correlate of protection and the more conservative threshold of ≥128 for long term protection. [13-18].

Statistics

No formal sample size calculation was performed, as only a proof of principle was required. Sample sizes for dose finding studies are usually small. The adaptive design allowed for dose switching at interim analysis, which optimized the use of subjects.

Ethical considerations

The study was approved by the local Medical Ethics Committee (METC) of the Leiden University Medical Center (LUMC, Leiden, the Netherlands) under NL41342.058.12 and EudraCT 2012-003085-41. All participants provided informed consent before inclusion and again before the booster.

3.4 Results

In total, 12 subjects were enrolled, all of whom had a negative verbal history for meningococcal vaccination including the meningococcal C mass vaccination campaign. Subjects were between 19 and 48 years of age at the time of inclusion and were equally divided between males and females (table 1). In the MenACWY-CRM197 arm, four subjects received a 1/10th dose. In the MenACWY-TT arm, four subjects received a 1/10th dose and four received a 1/5th dose fractional dose. Wheal size after intradermal injection ranged from 7 to 10 mm in diameter (mean 8.8 mm).

Table 1: Subject demographics

	MenACWY-CRM197 1/10th	MenACWY-TT 1/10th	MenACWY-TT 1/5th
No. of subjects	4	4	4
Age (mean years, range)	24 (19-28)	29 (20-48)	22 (20-26)
Sex (no. male)	2 out of 4	2 out of 4	2 out of 4
BMI (mean kg/m^2, range)	20 (17-22)	21 (19-24)	24 (21-26)
Concomitant vaccines	None	None	None
Smoking / drugs	NA	NA	NA
Race / ethnicity	NA	NA	NA

NA: data not available.

There were no serious adverse events (SAE). Adverse events were mild and generally short-lived (table 2). Two persons developed a notable adverse event (both in the MenACWY-TT arm): one subject developed a mild cheilitis which was diagnosed as a local reaction related to consumption of certain foods. The subject was initially treated with desloratadin and symptoms slowly resolved over the course of two weeks. The other subject developed a subtle papular rash on the trunk two weeks after both vaccinations but only reported it after the booster dose. The rash was less pronounced the second time and slowly faded over the course of three weeks. This last event was reported to the Netherlands Pharmacovigilance Centre (Lareb).

Table 2: Adverse events (AEs)

	Local erythema	Local pain	Headache	Fatigue	Myalgia
No of subjects (%)					
Primary dose	7 (58)	4 (33)	4 (33)	6 (50)	2 (17)
Booster dose	5 (42)	2 (17)	0 (0)	3 (25)	1 (8)

Table 3: Seroprotection rates

(seroprotected / total)	MenACWY-CRM197 1/10th	MenACWY-TT 1/10th	MenACWY-TT 1/5th
Meningococcus A			
Baseline (inclusion)	1 / 4	1 / 4	2 / 4
28d post primary	3 / 4	4 / 4	4 / 4
Prebooster (4-6 mo)	2 / 4	4 / 4	4 / 4
7d post booster	4 / 4	4 / 4	4 / 4
28d post booster	4 / 4	4 / 4	4 / 4
Meningococcus C			
Baseline (inclusion)	2 / 4	1 / 4	3 / 4
28d post primary	4 / 4	4 / 4	4 / 4
Prebooster (4-6 mo)	3 / 4	3 / 4	4 / 4
7d post booster	4 / 4	4 / 4	4 / 4
28d post booster	4 / 4	4 / 4	4 / 4
Meningococcus W			
Baseline (inclusion)	1 / 4	1 / 4	0 / 4
28d post primary	4 / 4	4 / 4	4 / 4
Prebooster (4-6 mo)	2 / 4	4 / 4	4 / 4
7d post booster	4 / 4	4 / 4	4 / 4
28d post booster	4 / 4	4 / 4	4 / 4
Meningococcus Y			
Baseline (inclusion)	1 / 4	2 / 4	1 / 4
28d post primary	4 / 4	4 / 4	4 / 4
Prebooster (4-6 mo)	4 / 4	4 / 4	4 / 4
7d post booster	4 / 4	4 / 4	4 / 4
28d post booster	4 / 4	4 / 4	4 / 4

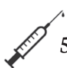
Definition of seroprotection: titer >=1:8 (rSBA).

At inclusion (baseline) there was pre-existing immunity against at least 1 vaccine serogroup in all subjects. Six subjects showed (mostly low) levels of pre-existing immunity against serogroup C, 4 subjects against serogroup Y, 4 against serogroup A, and 2 against serogroup W (table 3). There was no correlation between the presence of protective antibodies at baseline and the occurrence of adverse events.

One month after the fractional intradermal dose, the 1/10th dose of MenACWY-CRM197 had resulted in protective antibody levels in the rSBA assay in all subjects against all serogroups, with the only exception of meningococcal A antibody levels, which showed seroprotection in only 3 out of 4 subjects (table 3). Both the 1/5th and 1/10th doses of MenACWY-TT resulted in protective antibody levels in all subjects against all serogroups. It should be noted that dose escalation decisions were made on MIA data (interim serology, not reported here), not on rSBA data. For this reason, the MenACWY-TT arm was escalated despite the good performance of the 1/10th dose as reported here.

Geometric mean titers one month after the fractional intradermal dose are shown in figure 1 and the supplemental table, revealing a possible dose-response relationship and higher titers in the group vaccinated with MenACWY-TT when compared to MenACWY-CRM197. Four to six months after the primary vaccination (at the pre-booster baseline), antibody levels had decayed more in the MenACWY-CRM197 group than in the MenACWY-TT group (figure 1 and supplemental table). Antibody decay resulted in a reduction in seroprotection rates for serogroups A, C, and W in the MenACWY-CRM197 1/10th group and serogroup C in the MenACWY-TT 1/10th group. In the MenACWY-TT 1/5th group, all subjects remained seroprotected against all serogroups in the vaccine (figure 1 and supplemental table).

Seven days after the booster dose, all subjects were seroprotected against all vaccine serogroups. Twenty-eight days after the booster dose, antibody levels had not changed significantly compared to 7 days post booster. The memory response after boosting as measured by fold increase in GMT was more substantial in the MenACWY-CRM197 group than in the MenACWY-TT groups, perhaps due to lower initial antibody levels and faster decay in the MenACWY-CRM197 group (figure 1).



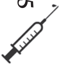
Supplemental Table 1: Detailed serologic results including GMTs with CI and range

		Menveo 1/10th			Nimenrix 1/10th			Nimenrix 1/5th		
			upper/max	lower/min		upper/max	lower/min		upper/max	lower/min
MenA										
baseline	SBA GMT (RSD, 95% CI)	5,7	43,4	0,7	9,5	131,1	0,7	45,3	704,6	2,9
	range		128	2		512	2		512	4
	Already seroconverted	1 / 4			1 / 4			2 / 4		
28d post	SBA GMT (RSD, 95% CI)	128,0	2049,2	8,0	1448,2	3480,7	602,5	2048,0	7968,0	526,4
	range		1024	2		4096	512		4096	256
	Seroconversion	3 / 4			4 / 4			4 / 4		
preboost	SBA GMT (RSD, 95% CI)	38,1	730,3	2,0	608,9	1431,3	259,0	2435,5	4667,0	1271,0
	range		512	2		2048	256		4096	1024
	Seroconversion	2 / 4			4 / 4			4 / 4		
7d post-b	SBA GMT (RSD, 95% CI)	512,0	1121,8	233,7	1217,8	3884,9	381,7	2896,3	4287,2	1956,7
	range		1024	256		4096	256		4096	2048
	Seroconversion	4 / 4			4 / 4			4 / 4		
28d pb	SBA GMT (RSD, 95% CI)	861,1	2024,2	366,3	1448,2	2856,4	734,2	2896,3	4287,2	1956,7
	range		2048	256		4096	1024		4096	2048
	Seroconversion	4 / 4			4 / 4			4 / 4		
MenC										
baseline	SBA GMT (RSD, 95% CI)	11,3	107,7	1,2	4,0	15,6	1,0	32,0	406,4	2,5
	range		256	2		32	2		1024	2
	Already seroconverted	2 / 4			1 / 4			3 / 4		

		Menveo 1/10th			Nimenrix 1/10th			Nimenrix 1/5th		
			upper/max	lower/min		upper/max	lower/min		upper/max	lower/min
28d post	SBA GMT (RSD, 95% CI)	1217,8	7905,9	187,6	5792,6	32011,0	1048,2	13777,2	43952,8	4318,6
	range		8192	128		32768	512		65536	4096
	Seroconversion	4 / 4			4 / 4			4 / 4		
preboost	SBA GMT (RSD, 95% CI)	256,0	4098,4	16,0	181,0	7629,8	4,3	4096,0	12419,6	1350,9
	range		2048	4		8192	2		16384	1024
	Seroconversion	3 / 4			3 / 4			4 / 4		
7d post-b	SBA GMT (RSD, 95% CI)	608,9	6994,9	53,0	1722,2	7766,3	381,9	5792,6	13922,9	2410,0
	range		4096	16		8192	256		16384	2048
	Seroconversion	4 / 4			4 / 4			4 / 4		
28d pb	SBA GMT (RSD, 95% CI)	724,1	14725,4	35,6	2435,5	7769,8	763,4	4871,0	9334,0	2542,0
	range		8192	8		8192	512		8192	2048
	Seroconversion	4 / 4			4 / 4			4 / 4		
MenW										
baseline	SBA GMT (RSD, 95% CI)	8,0	78,7	0,8	6,7	72,5	0,6	2,0	2,0	2,0
	range		256	2		256	2		2	2
	Already seroconverted	1 / 4			1 / 4			0 / 4		
28d post	SBA GMT (RSD, 95% CI)	476,5	1141,1	198,9	2048,0	2048,0	2048,0	4096,0	7132,4	2352,3
	range		1024	192		2048	2048		8192	2048
	Seroconversion	4 / 4			4 / 4			4 / 4		
preboost	SBA GMT (RSD, 95% CI)	38,1	769,0	1,9	724,1	2658,8	197,2	1448,2	2143,6	978,3
	range		1024	2		4096	256		2048	1024
	Seroconversion	2 / 4			4 / 4			4 / 4		

		Menveo 1/10th			Nimenrix 1/10th			Nimenrix 1/5th		
			upper/max	lower/min		upper/max	lower/min		upper/max	lower/min
7d post-b	SBA GMT (RSD, 95% CI)	608,9	1686,7	219,8	1722,2	3300,1	898,7	3444,3	6600,1	1797,4
	range		2048	256		4096	1024		8192	2048
	Seroconversion	4 / 4			4 / 4			4 / 4		
28d pb	SBA GMT (RSD, 95% CI)	861,1	2747,1	269,9	1448,2	2143,6	978,3	2435,5	3420,5	1734,1
	range		4096	256		2048	1024		4096	2048
	Seroconversion	4 / 4			4 / 4			4 / 4		
MenY										
baseline	SBA GMT (RSD, 95% CI)	5,7	43,4	0,7	24,3	425,2	1,4	5,7	28,5	1,1
	range		128	2		512	2		64	2
	Already seroconverted	1 / 4			2 / 4			1 / 4		
28d post	SBA GMT (RSD, 95% CI)	512,0	891,5	294,0	2896,3	4287,2	1956,7	5792,6	13922,9	2410,0
	range		1024	256		4096	2048		16384	2048
	Seroconversion	4 / 4			4 / 4			4 / 4		
preboost	SBA GMT (RSD, 95% CI)	362,0	714,1	183,5	1133,2	5866,8	218,9	4096,0	10704,5	1567,3
	range		1024	256		4096	96		8192	1024
	Seroconversion	4 / 4			4 / 4			4 / 4		
7d post-b	SBA GMT (RSD, 95% CI)	6888,6	60621,0	782,8	3444,3	8096,8	1465,2	4871,0	13493,7	1758,3
	range		65536	1024		8192	1024		8192	1024
	Seroconversion	4 / 4			4 / 4			4 / 4		
28d pb	SBA GMT (RSD, 95% CI)	2048,0	7078,5	592,5	2896,3	5712,9	1468,4	5792,6	18786,5	1786,1
	range		8192	512		4096	1024		16384	1024
	Seroconversion	4 / 4			4 / 4			4 / 4		

RSD = Reciprocal Serum Dilution



3.5 Discussion

To our knowledge, this is the first study evaluating the intradermal administration of a conjugate meningococcal vaccine. Our dose finding study demonstrates that fractional dose intradermal vaccination with conjugated quadrivalent meningococcal vaccine is sufficient to induce adequate seroprotection after a single dose, at least in our small group of subjects. Both fractional dose levels of MenACWY-TT resulted in complete seroprotection against all vaccine serogroups, whereas the 1/10th fractional dose of MenACWY-CRM197 resulted in less than complete seroprotection against serogroup A but complete protection against the other serogroups.

Pre-existing immunity at baseline was most pronounced against serogroup C (6 subjects), followed by serogroup A and Y (4 subjects each) and serogroup W (2 subjects each). In 3 subjects, there was pre-existing immunity against more than 1 vaccine serogroup. The presence of immunity against serogroup C at inclusion in 6 subjects (aged between 20 and 27) indicates that they had been vaccinated against meningococcal C disease even though they reported no previous meningococcal vaccination, since the diminished circulation of MenC due to the mass campaign in 2002 (coverage of 94%) has made it extremely unlikely that they would have encountered meningococcus serogroup C in the course of their daily lives in the Netherlands. As for the other serogroups, sporadic encounters are the most likely source of immunity, as most subjects were university students. This pre-existing immunity may impact our findings. It is unclear how the pre-existent immunity influenced vaccine response because the presence of immunity at the start of the study did not seem to correlate with post-vaccination antibody levels, as might have been expected in case of a recall response.

Post-primary vaccination antibody levels were higher in those subjects vaccinated with MenACWY-TT as compared to MenACWY-CRM197. This finding is consistent with data published by Diez-Domingo [19, 20], who demonstrated that meningococcal C vaccines conjugated to tetanus toxoid showed superior GMTs one month post vaccination and longer antibody persistence compared to vaccines conjugated to CRM197. These findings should be taken into account when evaluating national vaccination programs[21], since loss of seroprotection over time could be more substantial in those vaccinated with MenACWY-CRM197 than with MenACWY-TT. After the mass meningococcal C vaccination in the Netherlands in 2002 (of note: with a TT-conjugated vaccine and a coverage of 94%) herd effects were substantial and were shown to last >10 years[22].

Antibody levels decayed significantly in the MenACWY-CRM197 arm during the 4-6 months

between primary vaccination and the booster dose. Revaccination after 4-6 months generally did not substantially boost antibody levels for MenACWY-TT, but did result in a substantial boosting in the MenACWY-CRM197 group in which antibody levels had already decayed. Even though satisfactory seroprotection rates were achieved in this study after primary vaccination, booster doses might be necessary in order to extend the duration of protection. Ravenhorst et al. modelled antibody decay after boosted intramuscular vaccination against serogroup C and showed that the median time to fall below the protective threshold was around 270 years in the oldest subjects (15 years old) [23]. In intradermal vaccination, a relatively shorter duration of protection may be expected due to lower post-vaccination GMT as was noted after fractional rabies vaccination [24]. The same principle could apply to meningococcal vaccines, especially if boosting through natural exposure is reduced by widespread adoption of the vaccine.

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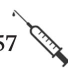
In this study the optimal timing of revaccination has not been studied. The duration of protection may be longer if revaccination is done after one year instead of six months. Future studies should take the aforementioned into account and 1) include a long term follow-up sample (at least ≥1 year after the primary vaccination) and 2) delay boosting to achieve the maximum response.

All serologic samples in this study were first evaluated in the Luminex MIA in the interim analysis and after the study had finished also in the serum bactericidal assay (SBA). The MIA measures serogroup specific anti-meningococcal polysaccharide IgG antibodies, while SBA is a functional assay. As such, effects of IgM and other immune system components besides IgG are measured in the SBA and may account for the difference between MIA and SBA in rate of protection which led us to administer booster doses after interim analysis. Furthermore, the MIA used here only measured antibody concentration, not avidity or bactericidal activity, both of which differ markedly between IgG subclasses.[25]

Our study did not include an intramuscular comparator arm. Using 'historical' controls from the a cross-sectional population based seroprevalence study (conducted in the Netherlands every 10 years by the National Institute of Public Health and the Environment RIVM), the intradermal regimes tested here for both vaccines show roughly the same antibody levels against serogroup C as the levels that were achieved with intramuscular administration as part of the Dutch national immunization program [26]. Antibody levels against serogroups A, W, and Y were also comparable to those reported by Ravenhorst et al after intramuscular vaccination with quadrivalent vaccine,



although in that study participants were slightly younger[17]. It should be noted that antibody decay appeared to be much more pronounced in the 1/10th group than the 1/5th group. In the latter group, the prebooster antibody levels were comparable to those found by Ravenhorst one year after a standard intramuscular dose [18].

This study marks the first trial to evaluate fractional dose intradermal administration of a conjugated vaccine. As such, local reactogenicity was followed-up with extra care and a data monitoring safety board (DSMB) was included in our study design. Because adverse events were negligible, the DSMB was discontinued for the booster phase of the study.

In contrast to conjugated meningococcal vaccines, unconjugated meningococcal polysaccharide vaccines have previously been administered intradermally by Guerin et al. in 750 Ugandan children aged 2-19 [27]. In this study, both 1/5th and 1/10th fractional doses were evaluated using rSBA and non-inferiority was shown for serogroups W and Y for both doses, but for serogroup A only the 1/5th dose was non-inferior. For serogroup C, non-inferiority was not shown for any of the fractional doses. Interestingly in 2011, Barnes et al. showed that avidity was actually better after fractional dosing of polysaccharide meningococcal vaccine, but equalized after 1-year revaccination [25].

In conclusion, intradermal vaccination with quadrivalent conjugate meningococcal vaccine appears to be safe and effective in our dose finding study. Confirmation in larger cohorts is required. TT-conjugated vaccine induced higher antibody levels compared to CRM197-conjugated vaccine, and 1/5th fractional dosing is preferable to 1/10th. Future studies should include an intramuscular comparator arm and long-term follow-up of antibody level decay before (booster) revaccination.

Declarations of interest:

None. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author contributions

LV and EJ conceived of and designed the study. EJ executed the study under supervision of LV. GB and MR performed the serological analyses. Subsequent interpretation of the data was performed by all authors. EJ drafted the article and it was critically revised for important intellectual content by LV, GB and MR. All authors have approved the final article.

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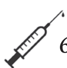

Chapter 4

Comparison of the PRNT and an immune fluorescence assay in yellow fever vaccinees receiving immunosuppressive medication

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4.1 Abstract

Background

The 17D-yellow fever (YF) vaccination is considered contraindicated in immune-compromised patients; however, accidental vaccination occurs. In this population, measuring the immune response is useful in clinical practice.

Methods

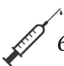
In this study we compare two antibody tests (the Immune Fluorescence Assay and the Plaque Reduction Neutralization Test) in a group of Dutch immune-compromised travellers with a median of 33 days (IQR [28-49]) after primary YF vaccination.

Results

We collected samples of 15 immune-compromised vaccinees vaccinated with the 17D yellow fever vaccine between 2004 and 2012. All samples measured in the plaque reduction neutralization test yielded positive results (>80% virus neutralization with a 1:10 serum dilution). Immune Fluorescence Assay sensitivity was 28% (95% CI [0.12-0.49]). No adverse events were reported.

Conclusions

All immune-compromised patients mounted an adequate response with protective levels of virus neutralizing antibodies to the 17-D YF vaccine. No adverse effects were reported. Compared to the plaque reduction neutralization test, the sensitivity of the Immune Fluorescence Assay test was low. Further research is needed to ascertain that 17D vaccination in immune-compromised patients is safe.



4.2 Background

Yellow fever virus and vaccine

Yellow fever (YF) is a potentially lethal viral disease caused by an RNA virus belonging to the Flaviviridae. In 1937, the live attenuated 17D-YF vaccine was developed. The vaccine has been proven safe and very effective: in nearly all studies, virus neutralizing antibodies develop in more than 90% of vaccinees; and antibodies appear to persist for several decades [1-3], if not lifelong [4]. Currently, international guidelines recommend vaccination against YF from nine months of age, for people traveling to or living in YF-endemic areas. After YF-vaccination, vaccinees have reported mild and transient adverse events, including fever, headache and local pain [5, 6].

Serious adverse events (SAEs) rarely occur in healthy vaccinees. Around 0·3-0·4/100.000 vaccinees develop yellow fever vaccine associated viscerotropic disease (YEL-AVD), which resembles the clinical course of wild-type YF infection. An estimated 0·4-0·8/100.000 develop yellow fever vaccine-associated neurotropic disease (YEL-AND), which presents with various clinical neurological symptoms, such as Guillain Barré Syndrome and encephalitis. Anaphylactic reactions have been estimated to occur in 0·8-1·8 per 100.000 vaccinations [7-9].

Immune-compromised individuals and the 17D-YF vaccine

In immune-compromised patients, there may be an increased risk of serious adverse events (SAEs) such as YEL-AVD and YEL-AND [10, 11]. To date, the exact pathophysiological mechanism of these SAEs has not been elucidated. Possibly, they are the result of an impaired immunologic response in the host, resulting in increased viral replication. In addition to the risk of SAEs, protection against YF after vaccination may be inadequate. For example, in HIV positive patients with low CD4 counts and high viral loads, virus-neutralizing antibodies (VNAs) were less persistent and geometric mean titers (GMTs) were lower [12-14].

YF vaccination in immune-compromised patients is contraindicated in existing guidelines. Consequently, to date, no studies exist that investigate early antibody responses among patients using immunosuppressive medication. Occasionally, immune-compromised patients receive YF-vaccination accidentally, e.g. because certain immune-suppressants are not recognized as such, or because of incomplete history taking.

Two small studies have described that adverse events did not occur more frequently in this patient group compared to healthy vaccines. However, with 70 and 19 patients included, these studies were not sufficiently powered to identify rare serious adverse events. Also, it is not clear whether or not these patients were protected by neutralizing antibodies from earlier YF vaccinations [15, 16].

Available tests and cross reactivity

Various serologic tests are available to measure the presence and amount of VNAs in vaccinees. Currently, the Plaque Reduction Neutralization Test (PRNT) is considered as gold standard [17, 18]. More commonly, however, the Indirect Immune-fluorescence Assay (IFA, EuroImmun (Lübeck, Germany)) is used, because of lower costs and processing time and because the PRNT was not routinely available for clinical use.

The IFA test for IgG antibodies has been found to be highly sensitive, but not specific compared to the PRNT in an early study by Monath et al [18]. An explanation for lower specificity is that cross-reactions between various flaviviridae can occur with IFA, resulting in more false positive results [19, 20]. According to a more recent study that comprised 150 serum samples, the IgG IFA using EuroImmun Biochip technology had a high sensitivity and specificity (both 95%) compared to the PRNT as gold standard [21].

Study objective

In the past years, we occasionally encountered immune-compromised travellers from various hospitals across the country (including our own) who had been accidentally vaccinated against YF despite having an absolute contra-indication. In addition, several patients received YF-vaccinations in our clinic not by accident, but because they had planned to travel to highly endemic areas, despite medical advice not to do so. In these situations, an individual decision whether or not to vaccinate was made, weighing the risk of SAEs (depending on the time interval, dose and type of medication used) against that of acquiring yellow fever (based on traveler vaccination guidelines and previous risk estimates [22, 23]). We therefore had the unique opportunity to study the immune response in this group of patients. To gain insight into the immunologic response in this population, we tested the hypothesis that patients using immunosuppressive medication would have a sub-optimal immunologic response to the 17D-YF vaccine.

4.3 Methods

Ethics

The protocol and consent forms for this study were approved by the Medical Ethics Committee of the Academic Medical Center (MEC AMC).

Recruitment of samples

We included all available post vaccination samples from travellers using immunosuppressive medication who had been vaccinated for the first time with a 17-D-204 YF vaccine (Stamaril or Arilvax) between 2004 and 2012. We collected demographic data (age, sex) and clinical data (medical history, time interval, type and dose of medication, previous vaccinations, adverse events, days between last vaccination dose and sampling). Patients who had stopped the immunosuppressive medication ≥ three months prior to vaccination were excluded. Additionally, we analyzed stored sera of healthy vaccinees who had been vaccinated subcutaneously between 2005 and 2007 with a comparable time interval between vaccination and blood sampling. These sera were stored at -20 °C from sampling until determination of the NAb titer.

Adverse events

Adverse events were self-reported. A physician was available 24/7 in case of adverse events following vaccination.

Serology

Immune Fluorescence Assay (IFA)

Serum samples taken approximately one month or longer after YF 17-D vaccination were sent to the Department of Virology at the Erasmus Medical Center Rotterdam, the Netherlands, to measure yellow fever IgG responses by IFA using the EuroImmun assay. Sera were diluted in 1:10, 1:32, 1:100, 1:320, and 1:1,000 and incubated on a biochip with YFV infected cells. Biochips are glass slides with YFV-infected cells cut into millimeter-sized fragments, after fixation and gamma-irradiation [21]. Incubation was performed using the Titerplane technique, allowing all Biochips to come into contact with the reagents simultaneously [21]. Sera which reacted in the YFV IgG IFA with a titer of 1:100 were also analyzed for other flavivirus-specific antibodies as controls (tick-borne encephalitis

virus (TBEV) strain K32, West Nile virus (WNV) strain NY, and a Japanese encephalitis virus (JEV) strain). In the second reaction step, after 30 min of incubation at 20°C, the slides were washed with washing buffer before the incubation with the fluorescein isothiocyanate-conjugated anti-human IgG binds to the human antibody. Finally, after 30 min, the slides were washed again and covered by a cover slide before being analyzed using a fluorescence microscope at a wavelength of 488 nm. Titers of >1:100 for IgG were considered positive.

Plaque Reduction Neutralization Test (PRNT)

PRNTs were performed by the Department of Infectious Diseases at the Leiden University Medical Center (LUMC), where the test is routinely performed. The technique described by De Madrid and Porterfield (1969) was used, modified for the LUMC PRNT test setup [24]. Vero cells were seeded in six-well plates (Corning Inc., USA) and cultured to obtain a monolayer. Heat-inactivated post vaccination sera were tested in two-fold dilutions up to 1:8192, all assayed in duplicate. One hundred Plaque Forming Units (PFUs) of 17D-YF were added to each serum dilution. After one-hour incubation on ice, the mixtures of virus and serum were added to the Vero cell monolayers and incubated for one hour at 37°C. An Avicel overlay was added. The overlay plates were incubated for four days at 37°C, followed by removal of the overlay and adding formaldehyde (7%) for 60 minutes. After staining, the formed plaques were counted manually. Virus neutralization (VN) was calculated for each serum dilution (i) with the following formula: $VN_{(i)}$ = 100 – 100 * ([average number of plaques in the diluted post vaccination serum]/[average number of plaques in the negative controls]). Protection against YF was defined as the occurrence of at least 80% $VN_{(i)}$ in a 1:10 serum dilution ($PRNT_{80}$), as specified by WHO [25]. Endpoint titers were standardized and reported in IU/mL to facilitate comparison across studies, using the 1st International Reference Preparation of Anti-Yellow Fever Serum (National Institute for Biological Standards and Control, UK). IU titers were calculated according to a modified version of Kaerber's formula as described by Cohen et al [26]: sample titer in IU/mL = $PRNT_{80}$ x unitage constant, where the unitage constant is the linear correlation between the reference serum's $PRNT_{80}$ titer and the antibody concentration in IU/mL. A study from 1996 suggests that MTX has antiviral activity against yellow fever virus [27]. In order to assess whether MTX had an influence on neutralization, virus neutralization in unvaccinated patients using MTX was tested.

Data Analysis

We compared positive and negative IFA and PRNT outcomes, and determined IFA sensitivity among

the primo-vaccinees of which serology was assessed at ≥ 27 days after vaccination, with PRNT as gold standard. Exact confidence intervals were calculated. The correlation between the IFA serum dilution and the antibody titer by PRNT, dose of methotrexate and the serologic outcomes, and time interval between vaccination and the serologic outcomes were determined.

4.4 Results

We collected data from 15 patients and 12 healthy controls. An IFA and a PRNT were performed on serum samples drawn from around one month post vaccination. None of the vaccinees reported adverse events following vaccination.

Vaccinees

The demographic and clinical characteristics of the vaccinees are depicted in Table 1 and 2. Out of 15 patients whose serum was available, 11 (73%) were using MTX, two (13%) etanercept, one (7%) prednisolone and one (7%) leflunomide. Most vaccinees had a history of rheumatoid arthritis (n=7; 47%). Three of the 15 vaccinees (20%) had discontinued the immunosuppressive medication two to six weeks before vaccination in anticipation of vaccination (Table 1).

Test results

IFA and PRNT results are shown in Table 1. These samples were obtained at least three weeks after vaccination. According to the IFA, seven out of the 15 patient samples (47%) had an antibody titer above the threshold (≥1:100). In healthy individuals, no samples were positive when tested with the IFA. In the PRNT on the other hand, 15 out of 15 patient samples (100%) were positive as were 10 out of 12 samples (83.3%) of healthy vaccinees.

The overall sensitivity of the IFA test was 28% (95% CI [0.12-0.49]). In patients, it was 47% (95% CI [0.215 – 0.73]) and in healthy controls it was 0% (95% CI [0.0-0.31]).

No influence of MTX on virus neutralization was seen in the PRNT. In order to assess whether MTX had an influence on neutralization in this study, sera containing MTX concentrations that ranged from 0.005-1 µmol/L were tested in the PRNT. There was no correlation with the number of plaques induced by yellow fever 17D vaccine virus, confirming results from Neyts et al who found a minimum cytotoxic concentration of 1.98 µmol/L. Because MTX concentrations in sera of patients treated with low-dose MTX (e.g. for RA) are generally lower than 0.05 µmol/L [28], we concluded that

Table 1: Demographic and medical details and titer results of 15 patients vaccinated for the first time

Case	Age	Sex	Medication	Dose (mg)	Medical history	Days vaccination to serology	IFA titer	Interpretation	Second IFA (outcome, time (d))	PRNT IU/mL	PRNT interpretation	Second PRNT
1	62	M	MTX	10	AP	35	1:1000	Pos		4.31	Pos	
2	50	M	MTX	10	AP	28	1:10	Neg	Pos (173)	0.07	Pos	Pos
3	51	F	MTX	15	RA	21	<1:10	Neg	Neg (65)	2.12	Pos	Pos
4	39	F	MTX	15	AP	27	1:10	Neg		4.97	Pos	
5	44	F	MTX	15 **	RA	33	1:100	Pos		1.55	Pos	
6	29	F	MTX	22.5	RA	49	1:1000	Pos		0.64	Pos	
7 #	51	M	MTX	25	RA	96	1:10	Neg		0.09	Pos	
8	56	F	MTX	25	Psoriasis	28	<1:10	Neg		0.39	Pos	
9	47	F	MTX	30	RA	61	1:100	Pos		2.55	Pos	
10 #	35	F	MTX	-	Scleroderma	35	1:10	Neg		0.10	Pos	
11	43	F	MTX	10	Scleroderma	28	1:100	Pos		0.26	Pos	
12	23	F	Prednisolone	60	PG	1407	1:100	Pos		0.09	Pos	
13	38	F	Leflunomide	20	RA	27	<1:10	Neg	Pos (213)	0.50	Pos	Pos
14	22	F	Etanercept	50 *	Psoriasis	34	1:1000	Pos		0.86	Pos	
15	28	F	Etanercept	- ***	RA	29	1:10	Neg		1.19	Pos	

RA = rheumatoid arthritis, PG = pyoderma gangrenosum, AP = arthritis psoriatica, MTX= methotrexate; Doses: MTX dose is weekly, prednisolone dose = daily, leflunomide dose = daily, etanercept dose = weekly, AEs = adverse events. # Second vaccination administered * stopped 2 weeks before vaccination **stopped 5 weeks before vaccination ***stopped 6 weeks before vaccination RA = rheumatoid arthritis, PG = pyoderma gangrenosum, AP = arthritis psoriatica, MTX= methotrexate; Doses: MTX dose is weekly, prednisolone dose = daily, leflunomide dose = daily, etanercept dose = weekly, AEs = adverse events. # Second vaccination administered * stopped 2 weeks before vaccination **stopped 5 weeks before vaccination ***stopped 6 weeks before vaccination

Table 2: Demographic details and titer results of 12 healthy primary vaccinees

Control	Age	Sex	Days from vaccination to serology	IFA titer	Interpretation	PRNT IU/mL	PRNT interpretation
1	58	F	28	1:10	Neg	3.47	Pos
2	27	F	28	1:10	Neg	1.32	Pos
3	44	M	28	<1:10	Neg	0.00	Neg
4	37	F	28	<1:10	Neg	1.81	Pos
5	22	F	28	1:10	Neg	1.04	Pos
6	40	M	56	1:10	Neg	0.42	Pos
7	36	F	28	1:10	Neg	4.54	Pos
8	28	M	28	<1:10	Neg	1.45	Pos
9	48	F	56	<1:10	Neg	0.85	Pos
10	69	F	28	<1:10	Neg	0.00	Neg
11	28	F	28	<1:10	Neg	0.41	Pos
12	29	F	56	<1:10	Neg	0.85	Pos

Demographic details and titer results of 12 healthy primary vaccinees

there was no effect of MTX on virus neutralization. No correlation between the IFA dilution and the percentage of neutralization of the PRNT was found. No correlations between time-interval since vaccination or doses of methotrexate and the serology titers were found.

4.5 Discussion

On the basis of our analysis of 15 immune-compromised patients who received a primo-yellow fever vaccination, we provide further (yet limited) evidence against the hypothesis that patients using immunosuppressive medication would have a sub-optimal immunologic response to the 17D-YF vaccine.

All 15 patients had formed VNAs measured by PRNT. As expected, most (83.33%) healthy vaccinees had a protective titer. Although none of the patients had reported any adverse events, our patient sample is far too small to draw any conclusion on the issue of safety.

Medication used and PRNT outcomes

Eleven out of 15 patients (73%) were using 10-30 mg MTX/week and all developed protective antibodies after vaccination despite the fact that MTX inhibits proliferation of activated B or T cells. Previously, doses lower than 0·4 mg/kg/week have been proposed to be safe in response to

administration of a varicella vaccine [29]. However, varicella is a treatable infection and poses less of a risk than the SAEs that could potentially follow vaccination with 17D-YF vaccine. Moreover, the absorption in the proximal jejunum, renal tubular secretion and reabsorption of MTX are highly variable between patients [30]. The dosing threshold of MTX below which YF-vaccination could be safe should therefore be regarded with caution.

Two patients used etanercept, one leflunomide and one prednisolone. They developed protective antibodies, despite the influence on the immune response through an altered cytokine milieu (anti-TNFα), limited DNA and RNA synthesis (DMARDs) or reduced cellular migration toward inflammation (corticosteroids) respectively.

IFA and PRNT test outcomes

We found large discrepancies between IFA and PRNT outcomes. Only 47% of patients were positive in the IFA and all healthy vaccinees tested negative, resulting in a very low overall sensitivity of 28%. Although we did not find a correlation between the IFA dilutions and the antibody concentrations found in PRNT, such a correlation has been described before (between GMTs of the IFA and the PRNT) [21, 31]. Our small group size could explain the lack of a correlation.

Based on the above, we conclude that the EuroImmun test as currently in use in the Netherlands is not suitable for assessing post vaccination seroprotection.

Reasons for unexpected outcomes

A possible explanation for the unexpected low sensitivity of the IFA is that the cut-off is too high. Antibodies present would have been picked up with a lower cut-off. For example, with a cut-off value of 1:10, five (instead of zero) out of 15 (33%) of healthy vaccinees would have been positive by IFA, increasing overall sensitivity to 68.0% [CI 46-84%].

A second explanation could be that one month between vaccination and serum collection (as was the case for most samples) was too short for IgG detection by IFA. In the IFA, fluorescent light is only emitted when serum antibodies (IgG) bind to the cell surface of pre-infected cells, and labeled anti-human antibodies bind to IgG. In the PRNT, subjects' serum is added to and reacts with the 17D YF vaccine strain and the earlier IgM response is measured as well. Although one study showed high rates of positive IgG antibody responses in samples collected 28 days after vaccination [21], possibly, IgG levels take longer than one month to rise to a detectable level. This was illustrated by two patients

who were IFA negative / PRNT positive after 27 and 28 days post vaccination, but IFA positive / PRNT positive after 173 and 213 days post vaccination (Table 1).

A third possibility could be that the PRNT yielded false-positive results, despite its definition as gold standard. Hypothetically, neutralization of the virus can be accomplished by components other than neutralizing antibodies, e.g. virus neutralizing medication, such as methotrexate present in the sera. However, we tested this hypothesis in our laboratory and found no effect of MTX on YF neutralization (unpublished results).

Revaccinated patients

Two out of 15 patients had received revaccinations after a negative IFA result. These revaccinations were unnecessary as PRNT outcomes were positive. The significance of a reliable test for post-vaccination titer control is illustrated by these cases.

4.6 Conclusions

Primary YF vaccination was immunogenic in a limited number of immune-compromised vaccinees. In those subjects, PRNT is a better test to measure yellow fever neutralizing antibodies compared to IFA. This unique dataset, although small, shows that from one month after vaccination with the YF-17D vaccine, protective levels of neutralizing antibodies were established in all patients, as measured with PRNT. The sensitivity of IFA was disappointingly low (28% (95% CI [0.12-0.49])), when compared to PRNT as gold standard. Based on these results, we conclude that PRNT should be used to assess post vaccination seroprotection, although larger patient numbers would be necessary to confirm our findings.

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4.7 References

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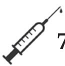

Chapter 5

A single 17D yellow fever vaccination provides lifelong immunity; characterization of yellow-fever-specific neutralizing antibody and T-cell responses after vaccination

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5.1 Abstract

Introduction

Prompted by recent amendments of Yellow Fever (YF) vaccination guidelines from boost to single vaccination strategy and the paucity of clinical data to support this adjustment, we used the profile of the YF-specific $CD8^+$ T-cell subset profiles after primary vaccination and neutralizing antibodies as a proxy for potentially longer lasting immunity.

Methods and Findings

PBMCs and serum were collected in six individuals on days 0, 3, 5, 12, 28 and 180, and in 99 individuals >10 years after YF-vaccination. Phenotypic characteristics of YF- $tetramer^+$ $CD8^+$ T-cells were determined using class I tetramers. Antibody responses were measured using a standardized plaque reduction neutralization test (PRNT). Also, characteristics of YF-tetramer positive $CD8^+$ T-cells were compared between individuals who had received a primary- and a booster vaccination.

YF-$tetramer^+$ $CD8^+$ T-cells were detectable on day 12 (median $tetramer^+$ cells as percentage of $CD8^+$ T-cells 0.2%, range 0.07-3.1%). On day 180, these cells were still present (median 0.06%, range 0.02-0.78%).

The phenotype of YF-tetramer positive $CD8^+$ T-cells shifted from acute phase effector cells on day 12, to late differentiated or effector memory phenotype ($CD45RA^{-/+}CD27^-$) on day 28. Two subsets of YF-tetramer positive T-cells ($CD45RA^+CD27^-$ and $CD45RA^+CD27^+$) persisted until day 180. Within all phenotypic subsets, the T-bet: Eomes ratio tended to be high on day 28 after vaccination and shifted towards predominant Eomes expression on day 180 (median 6.0 (day 28) vs. 2.2 (day 180) p=0.0625), suggestive of imprinting compatible with long-lived memory properties. YF-tetramer positive $CD8^+$ T-cells were detectable up to 18 years post vaccination, YF-specific antibodies were detectable up to 40 years after single vaccination. Booster vaccination did not increase titers of YF-specific antibodies (mean 12.5 vs. 13.1, p=0.583), nor induce frequencies or alter phenotypes of YF-$tetramer^+$ $CD8^+$ T-cells.

Conclusion

The presence of a functionally competent YF-specific memory T-cell pool 18 years and sufficient titers of neutralizing antibodies 35-40 years after first vaccination suggest that single vaccination may be sufficient to provide long-term immunity.

5.2 Introduction

Yellow fever (YF) infection is a continuous threat in endemic areas. It is characterized by a febrile disease, which, if jaundice occurs, can result in multi organ failure with a case fatality rate of up to 50% [1]. Because no curative treatment is available, only supportive care can be provided. Since the development of the 17-D YF vaccine in the 1930's, effective prevention is possible for people living in endemic areas and for those traveling to these regions. Current international regulations require a booster vaccination every 10 years. However, in May 2012, the Strategic Advisory Group of Experts [2] workgroup of the WHO proposed that revaccination every 10 years may not be necessary since lifelong immunity may be induced in most individuals with a single dose of YF vaccine [2, 3].

This proposed change in vaccination protocol has elicited debate because the clinical evidence on which the advice is based is limited [4, 5]. The optimal outcome measure for vaccination efficacy is the incidence of YF infections in vaccinated individuals. From 1942 until 2012, 12 cases of vaccine failure have been reported in vaccinated travellers [2]. The fact that vaccine failures did not correlate with an increasing time period since vaccination was used as an argument in favor of lifelong protection [2]. However, the number of vaccine failures was too small to draw firm conclusions regarding long-term protection without booster [2]. Given these limitations, characterization of the YF-specific immune response over time after a primary vaccination could help to provide further evidence for a single dose vaccination policy. YF vaccination has been shown to induce a vigorous YF-specific T cell as well as YF-specific antibody response [6, 7].

Upon vaccination, antigen specific antibodies of the IgM subclass are induced by day 7, reach a peak after 2 weeks, and are followed by the appearance of neutralizing YF-specific IgG antibodies (nAbs) [8]. The quantity of YF-specific nAbs wanes over time, but nABs have shown to remain detectable at 30 to 35 years after a single vaccination [9-11]. In addition to the neutralizing antibody response, YF-specific T-cells confer protection after 17-D YF vaccination [6, 12]. YF-tetramer positive CD8$^+$ T-cells appear in the peripheral blood 10-15 days after vaccination [13-16], and CD8$^+$ T-cells have been shown to complement nAbs in preventing YF infection after intracerebral challenge in a murine model [6, 11]. Taken together, protection against YF relies on the induction of neutralizing antibodies and may be further aided by YF-specific T cell responses. Insight into the long-term persistence and properties of this YF-specific immunity after single vaccination may be useful in supporting decisions on adjusting the vaccination scheme and are subject of this study.

CD8$^+$ T-cells display various phenotypic markers that correlate with functional properties. Classification of CD8$^+$ T-cells according to phenotype can help to make assertions about the ability to persist and respond to antigen re-challenge [17-22]. Early after antigen encounter, naive, YF-specific CD8$^+$ T-cells (CD45RA$^+$CD27$^+$CD28$^+$CCR7$^+$) are activated, undergo clonal expansion and differentiate to 'acute phase' T-cells (CD45RA$^-$CD27$^+$CD28$^+$CCR7$^-$) on day 14 after vaccination. These so-called 'acute phase' T-cells are cytotoxic, have down-regulated CD45RA, CCR7 and CD127 (IL-7Rα) but maintain high expression of CD27 and CD28. After the acute phase, on day 90 after vaccination, YF-specific T-cells develop into (CD45RA$^+$CD27$^+$CD28^{lo}CCR7$^-$) and (CD45RA$^+$CD27^{lo}CD28^{lo}CCR7$^-$) phenotypes which could be termed 'intermediately-differentiated' and 'late differentiated' phenotypes, respectively [15, 16]. The loss of CCR7, CD28 and CD27 during this differentiation occurs on antigen-experienced cells [17, 18, 20, 22-27] and is associated with gain of cytotoxicity [28].

In addition to the expression of cell surface markers and cytotoxic function, a distinction can be made between T-cell subsets through the expression of T-box transcription factors T-bet and eomesodermin (Eomes). T-bet and Eomes are key factors for differentiation and persistence of antigen-specific CD8$^+$ T-cells and their relative gene-expression level ultimately determine determine T-cell function. In naive cells, these transcription factors are minimally expressed but when cells are activated, expression increases [29]. Together, T-bet and Eomes cooperate to induce production of IFN-gamma, granzyme B and perforin [30-33]. T-bet drives the differentiation from naive towards an effector phenotype and is associated with high granzyme B and perforin presence [30, 34, 35]. On the other hand, lack of Eomes is associated with defects in long-term persistence and diminished secondary expansion upon rechallenge, suggesting that Eomes is associated with fitness of long-lived memory T-cells [31-33].

Earlier studies showed that up to 90 days after vaccination, YF specific CD8$^+$ T-cells are detectable in the circulation [15, 16]. However, it is unknown how long YF-tetramer positive T-cells are maintained and what their functional profile is, at such a late time after vaccination. Insight in these properties of YF-specific CD8$^+$ T cells may help to provide a rationale behind a single vaccination strategy.

In the present study, we performed a corroborative analysis of the frequencies and functional properties of YF-specific CD8$^+$ T-cells in a cohort of vaccinated healthy individuals up to 180 days after primary vaccination. In addition, we compared the frequency and properties of CD8$^+$ T-cells longer after primary versus booster vaccination (median 6.5 years, range 0-37 years), in order to

assess the effect of a booster vaccination on the long-term YF-specific $CD8^+$ T-cell response and the neutralizing antibody response.

5.3 Materials and Methods

Study population

The study population consisted of two groups. One group was prospectively enrolled to obtain PBMC at different time points following vaccination (n=6). These healthy volunteers were vaccinated against yellow fever (Stamaril®, Sanofi Pasteur MSD, Belgium) and PBMCs were obtained at days 0 (before vaccination), 3, 5, 12, 28 and 180 following vaccination. A separate group (retrospective) consisted of healthy volunteers (n=99), from whom serum was collected at a median of 16.0 years (range 11-40 years) after vaccination. In the latter group 96 had visited flavivirus endemic countries and 90 had visited yellow fever endemic countries. These individuals received either the Stamaril vaccine or Arilvax® (Novartis, UK) vaccine. Of these 99 individuals, in a subgroup (n=20), PBMCs were collected at a median of 6.5 years after vaccination (range 0-37 years) that all had visited yellow fever endemic areas. For both prospectively (singly vaccinated individuals) and retrospectively (both singly vaccinated and boosted individuals) collected PBMC's, tetramer stainings were performed as described below. Volunteers were recruited at the travel medicine centers of the Academic Medical Center, Amsterdam (AMC), the Leiden University Medical Center (LUMC) and the Havenziekenhuis, Rotterdam. Volunteers with an immune-compromising condition, an allergy to eggs or an age below 18 years were excluded.

Ethical approval

This study was approved by the Medical Ethics Committee of the Academic Medical Center, Amsterdam. Written informed consent was obtained from all volunteers.

PBMCs

PBMCs were isolated according to a standard protocol using density gradient centrifugation and were cryopreserved at -180°C until further use.

Tetramers

Tetramers were produced by the NIH Tetramer Core Facility at Emory University, Atlanta, USA: Five

immunodominant epitopes (NS4b 214-222 LLWNGPMAV, NS4a AMDTISVFL [15, 16], NS3 218-226 RRRLRTLVL [36], NS2b 110-118 HPFALLLVL [37], NS5 3178-3186 RPIDDRFGL [16] were loaded in BV450 labeled HLA-A02, HLA-A02, HLA-B27, HLA-B35 and HLA-B07 complexes, respectively.

Determination of phenotype of yellow fever specific CD8+ T-cells

For HLA-0A2, HLA-B35, HLA-B27 and HLA-B07 positive participants identified using polymerase chain reaction, yellow fever specific $CD8^{+}$ cells were identified using the tetramers described above. Twenty µL tetramer mix were added to 1-2 million cells per well in a 96-wells plate. After incubation for 30 minutes at 4°C, 30 µL of antibody mix including anti-CD3 V500 (BD Biosciences, (San Jose, CA, USA)), anti-CD8 BV785, anti-CD45RA BV650 from Biolegend (San Jose, CA, USA) anti-CD27 APC-eFluor 780 and anti-CD127 PE-Cy7 from eBioscience (San Diego, CA, USA) and Live/Dead fixable red cell stain kit (Invitrogen, Carlsbad, CA, USA) were added for 30 minutes. For intracellular staining, cells were fixated with the Fixation solution (eBioscience) for 20 minutes at room temperature and permeabilized with permeabilization solution (eBioscience). Cells were washed twice and a mix of

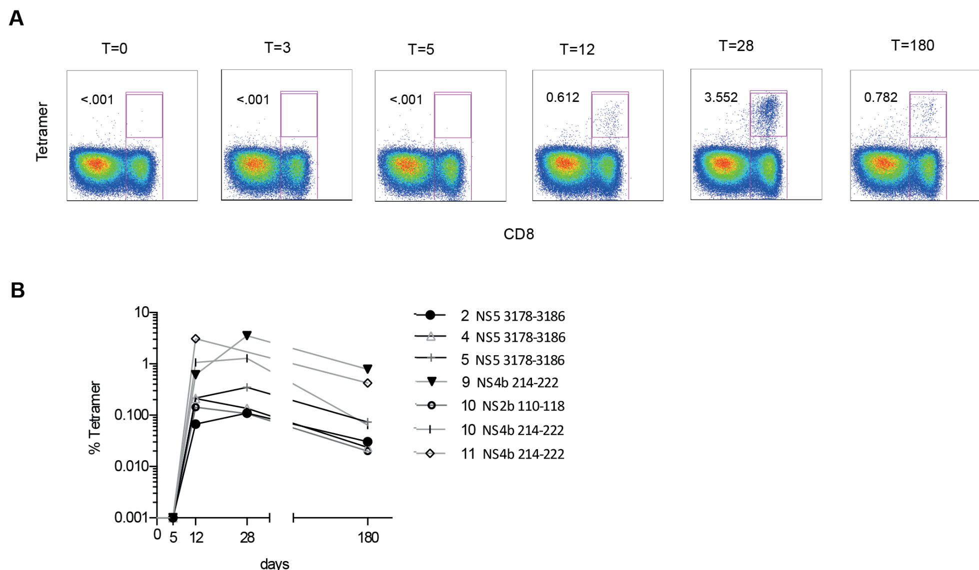

Figure 1: Frequency of YF-tetramer positive CD8+ T-cells in 6 singly vaccinated individuals (one individual (# 10) had 2 tetramer compatible HLA types, therefore seven lines are depicted). A. Dot plots of a representative donor B. Frequency of YF-tetramer+CD8+ T-cells expressed as percentage of YF-tetramer positive CD8+ T-cells directed against the NS2b, NS4b and NS5 epitopes in HLA-B35, HLA-A02 and HLA-B07 positive individuals at days 0, 3, 5, 12, 28 and 180 after vaccination.

intracellular antibodies comprising anti-Eomes PerCP-eFluor710 from BD Biosciences, anti-Ki67 BV711, anti-T-bet AF647 from Biolegend, anti-granzyme B AF700 from eBioscience, and anti-granzyme K PE from Immunotools (Friesoythe, Germany) was added for 30 minutes. Cells were then washed and re-suspended in 100 µl PBEA to be measured.

Gating strategy

Lymphocytes were gated using forward/sideward scatter properties. Duplets were excluded using forward scatter width/height- and sideward scatter (SSC) width/height characteristics. Dead cells were excluded using Live/Dead fixable red cell fluorescence intensity. $CD3^{+}CD8^{+}$tetramer^{+} events were gated as shown in Figure 1A. $CD8^{+}$ T-cell subsets were gated as $CD45RA^{+}CD27^{+}$, $CD45RA^{-}CD27^{+}$, $CD45RA^{+}CD27^{-}$ and $CD45RA^{-}CD27^{-}$ populations (Figure 2A). Granzyme K^{-} and Granzyme B^{+} and negative gates were gated as total $CD8^{+}$ and CD8+tetramer^{+} as shown in Figure 2B. T-bet and Eomes positive populations were gated as total $CD8^{+}$ and $CD8^{+}$tetramer^{+} as shown in Figure 3A.

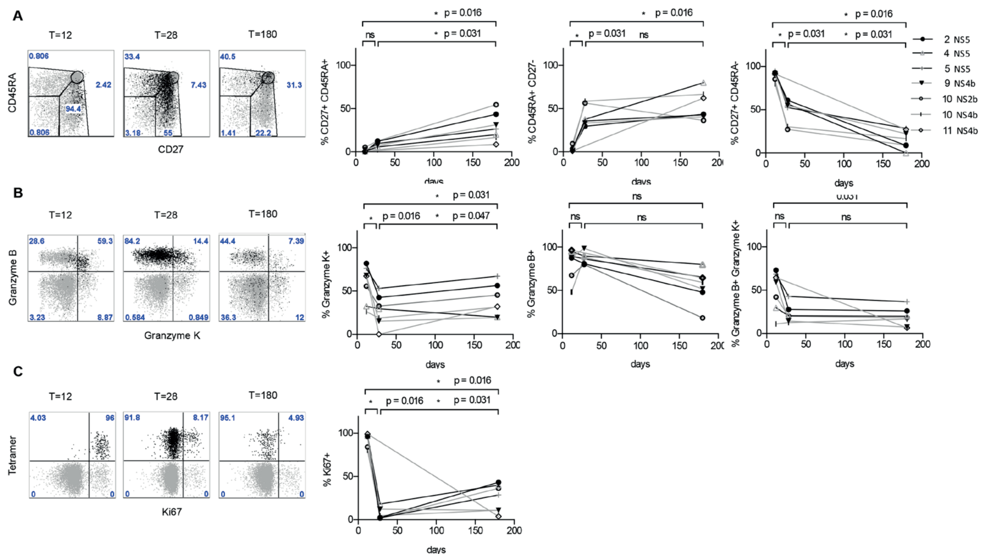

Figure 2: Longitudinal analysis of the phenotype of YF-tetramer positive CD8+ T-cells on days 12, 28 and 180 in singly vaccinated individuals. A Dot plots of a representative donor. Cells are gated on total CD8+ T-cells (in grey) and YF-tetramer positive cells (in black). B-D Summary of percentages of tetramer positive cells expressing CD45RA, CD27, granzyme K, granzyme B and Ki67 in 6 donors (1 donor had 2 matching HLA types). Comparisons were performed with a paired Wilcoxon Rank sum test. ns = not significant.

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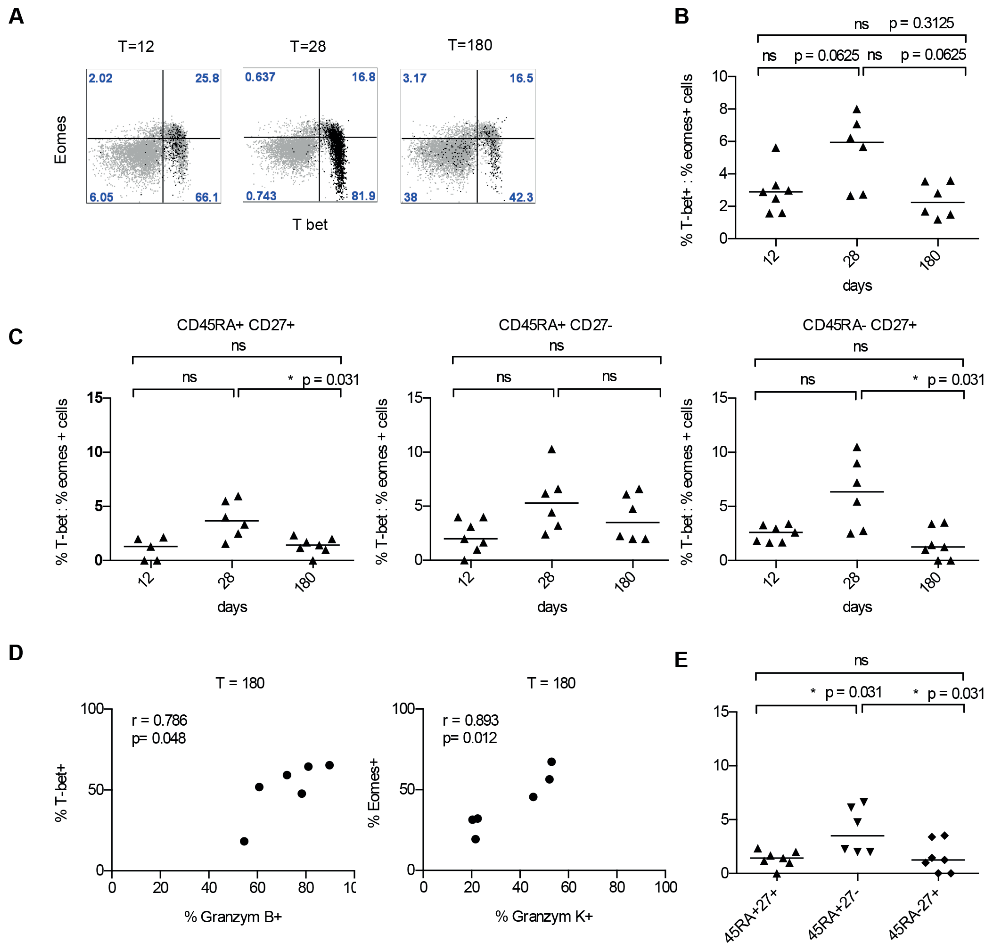

Figure 3: Longitudinal analysis of T-bet eomes expression in singly vaccinated individuals. A Dot plots of a representative donor. Total CD8+ T-cells are depicted in grey and YF-tetramer positive CD8+ T-cells in black. B-C T-bet:Eomes ratios on days 12, 28 and 180 in CD8+ tetramer+ cells. D Correlation between Granzyme K and Eomes expression and Granzyme B and Tbet expression on days 12, 28 and 180 after vaccination. E. T-bet:Eomes ratios of YFtetramer positive at T = 180 in different subsets. Comparisons were performed with a paired Wilcoxon Rank sum test. ns = not significant

Proliferation assay

In a number of donors of the retrospective group, YF-tetramer+ cells were not detectable ex vivo directly. We performed a proliferation assay to be able to detect low frequencies of YF-tetramer+ CD8+ cells. For this assay, PBMCs of HLA-A2, HLA-B35, HLA-B27 and HLA-B7 positive donors were labeled with carboxyfluorescein succinimidyl ester (CFSE) and cultured for 9 days in the presence

of YF peptides. PBMCs were cultured at 37°C and 5% CO2 in the presence of peptides corresponding the HLA type (0.1 µg/mL) in culture medium consisting of Iscove's Modified Dulbecco's Medium (IMDM) with 10% human pooled AB serum, penicillin/streptomycin and β-mercaptoethanol. Before culture, cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) to monitor cell division. Recombinant human interleukin-2 (IL-2) was added on days three and six in a concentration of 25 IU/mL. After 9 days, staining with YF-tetramers in combination with CD3, CD8 and viability dye was performed as described above.

Functional assay

For intracellular cytokine staining of YF-tetramer+ CD8+T-cells, PBMCs of tetramer reactive samples were stimulated for 6 hours with phorbol myristate acetate (PMA) and ionomycin. One to two million cells were incubated in medium consisting of RPMI-1640 with 10% fetal calf serum (FCS) in the presence of PMA (10 ng/mL) and ionomycin (1 ng/mL), anti-CD107a FITC (eBioscience), brefeldin A (10 microg/mL; Invitrogen), GolgiStop (BD Biosciences) and co-stimulation (anti-CD28) for 6 hours at 37°C and 5% CO2. As a control, the same conditions without PMA and ionomycin were used. After incubation, 20 µL of tetramer mix was added to the samples in a 96-wells plate for 30 minutes at 4°C. Subsequently, 30 µL of antibody mix with CD3 V500 (Invitrogen) and CD8 BV785 from Biolegend were added for 30 minutes. The Cytofix/Cytoperm reagent (BD Biosciences) was used for fixation and permeabilization. After permeabilization, the following monoclonal antibodies were added: anti-TNF-α AF700, anti-IL-2 PE (BD Biosciences), anti-Mip1-β PE-Cy7 (Biolegend), anti-IFN-γ APC-eFluor 780 (eBiosciences). Cells were analyzed by LSR Fortessa and FlowJo v. 9.7.6 software (Stanford University, 1995-1996).

Plaque reduction neutralization assays (PRNT)

For PRNTs the technique previously described by De Madrid and Porterfield (1969) was used, modified for the LUMC PRNT test setup [38]. In short, Vero cells were seeded in six-well plates (Corning Inc., USA) and cultured until a monolayer was formed. Heat-inactivated post-vaccination sera were tested in serial two-fold dilutions up to 1:8192. Pooled pre-vaccination sera were used as negative control. One hundred Plaque Forming Units (PFUs) of 17D-YF were added to each serum dilution. After one-hour incubation on ice, the mixtures of virus and serum were added to the Vero cell monolayers and incubated for one hour at 37°C, all assayed in duplicate. An Avicel overlay was added. The overlay plates were incubated for four days at 37°C, followed by removal of the overlay

and adding formaldehyde (7%) for 60 minutes, killing the virus and fixing the cell-layer. After fixation, 1 mL crystal violet solution was added for 10 minutes, staining only live cells. The plates were washed with water and were dried for one day. The formed plaques were counted manually. Virus neutralization (VN) was calculated for each serum dilution (i) with the following formula: $VN_{(i)} = 100 - 100 * ([\text{average number of plaques in the diluted post vaccination serum}]/[\text{average number of plaques in the negative controls}])$.

Protection against YF was defined as the occurrence of 80% $VN_{(i)}$ in a ≥1:10 serum dilution. The serum endpoint titer was defined as the reciprocal serum dilution in which 80% $VN_{(i)}$ occurred. Endpoint titers were also reported in IU/mL, using the 1st International Reference Preparation of Anti-Yellow Fever Serum (National Institute for Biological Standards and Control, UK).

Statistical analysis

Comparative analyses were performed using the Mann Whitney U test for continuous data and the Fisher's exact test for dichotomous data. Paired samples of non-parametric data were compared using the Wilcoxon Rank sum test. All T-tests were 2 tailed and $P<0.05$ was considered statistically significant. All analyses were performed in SPSS statistics v 19 (IBM, Chicago, IL, USA).

5.4 Results

YF-tetramer positive $CD8^+$ T-cells shift from acute phase phenotype on day 12 to a mixed population of $CD45RA^{hi}CD27^{lo}$ and $CD45RA^+CD27^+$ cells on day 180

Recent studies showed that vaccination induces YF-tetramer$^+$ $CD8^+$ T-cells and that they can be detected in the peripheral blood 10 days post-vaccination [15, 16]. In our study cohort, frequencies of YF-tetramer positive $CD8^+$ T-cells directed against three epitopes (NS4b 214-222, NS2b 110-118 and NS5 3178-3186) at 3, 5, 12, 28 and 180 days after first vaccination in 6 healthy HLA-A02, HLA-B35 or HLA-B07 positive donors were measured (Table 1). Day 12 was the first time point at which YF-tetramer positive $CD8^+$ T-cells were detectable (YF-tetramer$^+$ cells as percentage of $CD8^+$ T-cells: median 0.2%, range 0.07-3.1%) (Figure 1A). In accordance with earlier studies that showed that NS4b is an immune-dominant epitope [15], at day 28 after single vaccination, $CD8^+$ T-cells directed against the NS4b 214-222 epitope were present at significantly higher frequencies compared to $CD8^+$ T-cells directed against the other epitopes (mean 2.4% vs. 0.2%, $p = 0.037$) (Figure 1B).

Table 1: Demographic characteristics of participants in prospective follow up

Donor ID	Sex	Age (y)	HLA type
2	M	28	B07
4	F	42	B07
5	F	30	B07
9	M	46	A02
10	M	25	B35 and A02
11	F	22	A02

In analogy to previous studies, almost all (median 91.5%, range 79.9-94.7%) YF-tetramer positive $CD8^+$ T-cells had a high expression of $CD27^+$ and were $CD45RA^-$ on day 12 after vaccination, reflective of an acute phase effector phenotype (Figure 2A). At day 28 and day 180 the phenotype shifted towards a late differentiated or effector memory phenotype, marked by loss of CD27 and re-expression of CD45RA ($CD27^-CD45RA^+CD8^+$ T-cells, median 36.6%, range 29.6-58.6% on day 28 and median 43.5% range 36.4-80.0% on day 180). In addition to this effector memory population, on day 180, a $CD45RA^+CD27^+$ YF-tetramer positive population (median 26.5%, range 8.6-54.5%) was detectable (Figure 2A).

Taken together, as time since vaccination passes, YF tetramer$^+$ $CD8^+$ T-cells change from an 'acute phase effector' ($CD45RA^-CD27^+$) phenotype to a mixed population with a 'late or effector memory' ($CD45RA^+CD27^-$) and 'naive like' ($CD45RA^+CD27^+$) phenotype at day 180.

Late after vaccination, YF-tetramer positive $CD8^+$ T-cells are potentially cytotoxic

Virus-specific cells at different stages of differentiation vary in the expression of granzyme B and K. Granzyme K is expressed by early-differentiated cells and granzyme B is preferentially expressed by acute phase effector cells and late differentiated cells [39-41]. Granzyme K and B double positive tetramer positive cells are considered to represent a transitional form of $CD8^+$ T-cells from GrB^-/GrK^+ to GrB^+/GrK^- cells (early-differentiated cells transitioning to late-differentiated cells) [41].

To assess if YF-tetramer$^+$$CD8^+$ T-cells at day 180 are potentially cytotoxic, the expressions of granzyme B and K were determined. The expression of granzyme K within the YF-tetramer positive $CD8^+$ T-cell fraction significantly declined over time (p=0.031) in a biphasic pattern after single vaccination. After an initial decline in percentage of tetramer positive cells that express granzyme K from day 12 to day 28 (day 12 median 67.5%, range 26.2-82.1%; day 28 median 29.6%, range 0.0-53.1%, p=0.016), the percentage of granzyme K expressing cells in tetramer positive $CD8^+$ T-cells increased at day 180

(median 32.2%, range 19.4-67.3%; p=0.047) (Figure 2B). By contrast, the expression of granzyme B within tetramer positive CD8+ T-cells remained stable over time (p=0.078). Finally, granzyme K and B double positive tetramer positive cells tended to be highest at day 12 (median 59.3%, range 11.1-73.2%) and declined at day 180 (median 18.2%, range 6.4-36.7%, p=0.031) (Figure 2B). These data suggest that from day 12 on after vaccination, YF-tetramer+CD8+ T-cell have a cytotoxic potential that is maintained at least until 180 days post-vaccination. To further characterize the cytokine and chemokine profile of YF-tetramer+CD8+ T-cells, at all time points expression of TNF-α, Mip1-β, IL-2, IFN-γ and CD107a by YF-tetramer+CD8+ T-cells was analyzed. Over time, the fractions of cytokine and chemokine producing tetramer+ cells did not show significant changes (Supplemental File 1B). Overall, the majority of tetramer+ CD8+ T cells expressed 1 or more cytokines at day 12, 28 and 180 (Supplemental File 1C) and in all donors, cells were capable of expressing at least 4 cytokines, making them polyfunctional.

The ability of virus-specific CD8+ T-cells to persist relies on self-renewal capacity. To investigate whether YF-tetramer+CD8+ T-cells were indeed proliferating, the expression of Ki-67 as marker for active proliferation was determined (Figure 1C). At day 12 almost all YF-tetramer positive CD8+ T-cells were proliferating as reflected by the high percentage of cells expressing Ki-67 (median 96.4%, range 80.4-99.1%). After an initial decline of proliferating (Ki-67 positive) cells at day 28 (median 2.6%, range 0.0-18.2%; p=0.016) in three donors, the percentage of YF-tetramer+Ki67+ cells increased until from day 12 to day 180. In the other donors (n=3) the size of the Ki-67+ fraction remained constant. However, if all six donors were combined, the size of the Ki67+ fraction significantly increased over time (median 28.7% range 3.8-43.4%; p=0.031) (Figure 1C). In summary, YF-tetramer CD8+ T-cells maintain a cytotoxic potential, are polyfunctional and undergo homeostatic proliferation at least until 180 days after vaccination.

At 180 days after vaccination the T-bet:Eomes balance shifts in favor of Eomes in YF-tetramer positive CD8+ T cells.

Virus-specific cells that share phenotypic characteristics may be different with respect to their transcriptional profile [42]. T-box transcription factors T-bet and Eomes control the expression of proteins involved in effector function and homeostasis [43-45]. In this context, high T bet expression fosters the terminal differentiation of functional CD8+ T-cells [34, 46, 47] and Eomes is pivotal for sustaining memory subsets [31, 33]. In order to provide insight in the memory or effector potential

of YF-tetramer+CD8+ T-cells at the latest time point available after vaccination (day 180), the Eomes and T-bet expression ratio in the different T-cell subsets over time was determined in the YF-tetramer positive CD8+ T-cell fraction (Figure 3A). In total YF-tetramer+CD8+ T-cells at day 28 after single vaccination, we observed a trend towards an increase in the ratio of T-bet:Eomes, compared to day 12 (p=0.0625) and day 180 (p=0.0625) (Figure 3B). When YF-tetramer+CD8+ T-cells were separated according to phenotypic subset, as subdivided by CD45RA and CD27 expression, a similar trend in T-bet:Eomes ratio was found over time (Figure 3C). Taken together at day 180, both in total YF-tetramer+CD8+ T-cells as well in the different subsets, the Eomes expression tend to prevail above T-bet, suggestive of a potential capacity for long-lived memory cells. Finally, we investigated the association between T-bet and Eomes and granzyme B and K, respectively. The differentiation towards a CD27 negative phenotype is accompanied with gain of cytotoxicity / granzyme B [24] and early stage of T cell differentiation is associated with granzyme K upregulation [41].We found that T-bet expression positively correlated with granzyme B (Figure 3D) and Eomes expression correlated positively with granzyme K expression (Figure 3D).

The CD27+CD45RA+ cell subset present on day 180 is not naive

On day 180 after single vaccination we observed the presence of a CD27+CD45RA+ cell subset, classically compatible with a naïve function. In order to investigate the properties of this subset in more depth, the transcriptional profile as well as the expression of cytotoxic molecules was analyzed. Recently in YF-tetramer+CD8+ T-cells, mRNA profiling has shown that these naive-like cells were distinct from genuine naive cells and resembled stem cell like cells [48]. Indeed, YF-tetramer+CD8+ CD27+CD45RA+ cells showed low expression of granzymes B and K, similar to naive cells, but were CD28hi and only 50% expressed CCR7 (data not shown), confirming that these CD27+CD45RA+CD28+cells are at least partly antigen-experienced and may have re-expressed CD45RA [49].

Furthermore, in contrast to the expectation of a very low expression of T-bet and Eomes in naive cells [29], the expression of T-bet and Eomes was comparable between the CD27+CD45RA+ and the CD27+CD45RA- (early differentiated) population. On day 180, the CD45RA+CD27-, late-differentiated subset had a significantly higher T-bet:Eomes ratio compared to the CD45RA+CD27+ subset (p=0.031). The T-bet:Eomes ratio was comparable between the CD27+CD45RA+ and the CD45RA-CD27+, or early-differentiated subset (Figure 3E).

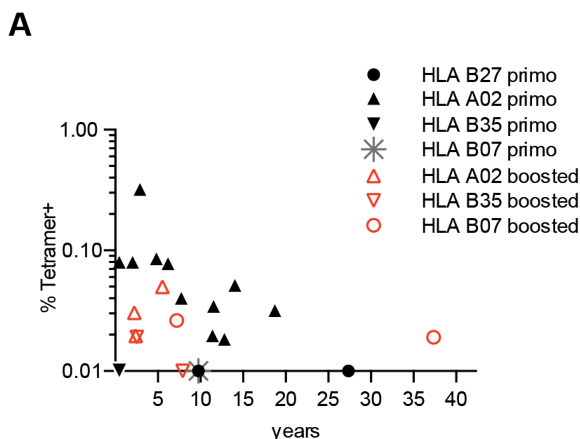

Figure 4: Percentages of YF-tetramer positive CD8+ T-cells over time in 13 healthy individuals that received a single vaccination of whom 11 were HLA A02, 2 HLA B27, 1 HLA B35 and 1 HLA B07 (2 donors had 2 HLA types compatible with tetramer reactivity). Seven donors, 3 HLA A02, 2 HLA B07 and 2 HLA B35 had received a booster vaccination. On the x-axis the number of years since last vaccination until PBMC collection is shown. On the y-axis the percentage of YF-tetramer+ cells gated on total CD8+ T cells is shown. Black, closed symbols depict single vaccinated individuals; red, open symbols depict boosted individuals. In 4 donors tetramer+ CD8+ T cells could not be detected directly ex-vivo but only after in vitro expansion by culturing for 9 days in the presence of IL-2 and a YF-peptide pool. Analysis of the correlation between YF-tetramer+ CD8+ T cells of singly vaccinated HLA-A2+ donors and time since vaccin ation showed a significant negative correlation ($r = -0.76$, $p = 0.0086$, Spearman's Rank Correlation Coefficient)

Overall it can be concluded that despite the high CD27and CD45RA expression, these CD8+ T-cells have T-bet:Eomes levels compatible with antigen-experienced cells.

A booster vaccination does not further induce the frequency and phenotype of YF-tetramer positive CD8+ T-cells

To evaluate whether booster vaccination leads to a further increase in frequencies or differentiation pattern of YF-tetramer positive CD8+ T-cells, the percentage of YF-tetramer positive CD8+ T-cells in 13 individuals who received a single vaccination in the past (median time since vaccination 10.0 years, IQR 3.0-13.3) was compared to seven individuals who received 1 or 2 boosters during their lifetime (median time since last booster 6.0 years, IQR [2.5-7.5]) (Supplemental File 2-3). The percentage of YF-tetramer positive CD8+ T-cells in the boosted group was comparable to the frequency of YF-tetramer

positive cells in those who were vaccinated only once (boosted group median 0.020% tetramer+ cells/ CD8+ cells, range 0.01-0.05%; primary vaccination group median 0.034% range 0.01-0.320%, p=0.365) (Figure 4). Also the phenotypic characteristics of YF-tetramer positive CD8+ T-cells (CD45RA+CD27-, CD45RA-CD27+, CD45RA+CD27+) of singly vaccinated and boosted individuals were comparable. Therefore, neither frequency nor phenotype of YF-tetramer positive CD8+ T-cells is influenced by multiple vaccinations.

YFV-neutralizing antibodies are present up to 40 years after vaccination

IgM and IgG YF-neutralizing antibodies are known to peak 2 and 4 weeks after vaccination, respectively, and decrease over time [8, 9, 12]. To investigate whether over time antibodies decreased below this threshold of protection in our population, we determined level of antibody in serum in 99 donors of whom serum samples were available at a median time of 16 years (range 11-40 years) after single vaccination (Supplemental File 4). In 89 out of 99 individuals (89.9%) antibody titers were detectable above the protective threshold (0.5 IU/mL). We observed that the height of the antibody titer correlated negatively with time since vaccination (r=-0.197, p=0.040). In addition, we analyzed the correlation between age and antibody titers and also found a negative correlation between antibody titers and age (r=-0.209, p=0.037, Spearman's rank correlation coefficient). However, we conclude that despite the decrease of titers with ageing, 90% of individuals still had protective levels of antibody. Furthermore, in a subgroup of 6 individuals of whom sera were available long after primary vaccination (35-40 years), antibodies were detectable at protective levels in all 6 individuals (median 60.5 IU/mL, range 2.30-83-90 IU/mL) (Figure 5). A booster vaccination did not result in higher antibody titers (median 5.1 vs. 9.4 IU/mL, p=0.583).

5.5 Discussion

In the present study, we characterized the long-term presence and functional profile of YF tetramer+CD8+ T-cells and nAbs as the two key immunological correlates of protection after single dose of YF vaccination. We showed that 180 days after primary vaccination CD45RA+CD27- late differentiated and CD45RA+CD27+, or 'naive-like' YF-specific cells were present, had a cytotoxic potential, were polyfunctional with respect to expression of cytokines profile, and showed a relatively low T-bet:Eomes ratio. Furthermore, 89/99 (89.9%) individuals vaccinated more than 10 years ago, and 6/6 individuals vaccinated 35-40 years ago had antibody levels in a range that is considered to

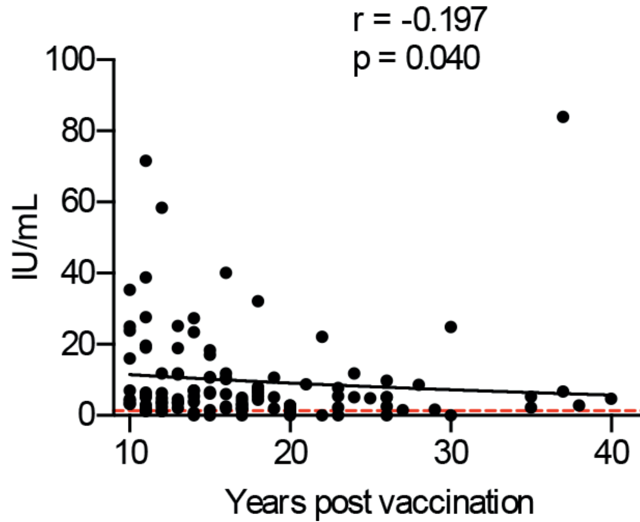

Figure 5: Correlation between YF-Antibody titers in 99 individuals that received a single vaccination and time since vaccination. The y-axis shows the time since vaccination and the x-axis shows the YF-serum antibody titer. The correlation between serum titer and time since vaccination was calculated with Spearman's Rank Correlation coefficient. As a reference, the red line depicts the YF antibody serum level threshold of protection (0.5 IU/mL)

be protective. Booster vaccinations did not further increase the frequencies of YF-tetramer positive CD8+ T-cells.

The generation of a long-lasting virus-specific T-cell response is key for long-term protection against infection. Prompted by the recent amendments of vaccination guidelines from boost to a single vaccination strategy and the paucity of clinical data to support this adjustment, we used the profile of the YF-specific T-cell subsets after primary vaccination as a proxy for potentially longer lasting immunity. We found that the frequencies of YF-tetramer positive CD8+ T-cells were comparable to those described in other studies [15, 16]. Our study adds to previous studies a long follow-up until 180 days and characterization of transcriptional profile of YF-specific CD8+ T cells at his late time point after vaccination. Although over time percentages declined, YF-tetramer+CD8+ T-cells were clearly detectable up to 18 years after vaccination. In earlier studies on YF vaccination in mice it was

shown that the presence rather than the quantity of YF-tetramer positive CD8+ T-cells is related to protection after vaccination [50]. Therefore, our observation of the presence of YF-tetramer+CD8+ T-cells very long after vaccination can be considered promising with regard to the duration of immunity against yellow fever.

The development of a virus-specific CD8+ T cell response is characterized by a clonal expansion of virus-specific cells which is followed by a contraction phase upon clearance or control of the virus. In the memory phase, different types of virus-specific cells persist with respect to differences in phenotype and functional profile. Several studies in the past years showed that the properties of memory cells are strongly associated with type of virus for which they are specific [18, 51]. This heterogeneity in memory T cells directed against different viruses is likely driven by external factors such as T cell receptor triggering and signaling and cytokine environment (reviewed in Wherry et al. Nat Rev Immunol 2014). Also, the ability of these external factors in shaping the type of effector and memory cell suggests that plasticity between subsets may exist. In this context, we showed, in line with other studies, that the phenotype of YF-tetramer+CD8+ T-cells differs from the classical memory phenotype of cleared viral infections such as influenza A [51-53, 18]. In the late stage of infection, or (in our study) long after vaccination, YF-tetramer+CD8+ T-cells for instance have a heterogeneous expression of CD28 and have re-expressed CD45RA, as is seen in CMV-specific late stage effector cells. This is in contrast to influenza [53] and RSV-specific CD8+ T cells [54] that have down regulated CD45RA. Furthermore, where FLU and RSV (cleared viruses) uniformly have a high CD27 expression, YF-specific CD8+ T cells show mixed populations with a high and low CD27 expression [14-16]. Taken together, the phenotype of the YF-tetramer+CD8+ T-cells has more characteristics of further differentiated, effector phenotype. In addition, we and others [15] show that YF-specific CD8+ T cells are polyfunctional, despite this population of apparently more differentiated phenotype. Taken together, these data indicate that at late time points after vaccination YF-specific CD8+ T cells do not fit in a "typical" memory or effector profile.

The YF-specific CD8+ T-cell pool consisted of two phenotypic different populations with a late-differentiation and naive-like phenotype, that both were polyfunctional and expressed granzyme B. In an earlier study we found that YF-tetramer+CD8+ T-cells 18 years after vaccination have a phenotype that resembles the subsets on day 180 in our present study [55].

Taken together, deducted from the phenotypic appearance and function, these findings support the

assumption that these subsets and their persistence as measured 180 days and 9 years after primary vaccination indeed may confer protection until many years later.

The characterization of the expression of the transcription factors Eomes and T-bet further deepens insight in the potential for longevity of vaccination induced YF-tetramer$^+$CD8$^+$ T-cells. In this context, the expression of Eomes is associated with longevity and effective proliferation upon reencountering antigen in mice [32-33]. CD8$^+$ T cells lacking Eomes are defective in long-term survival [31]. Furthermore, a recent study showed that the combination of phenotype and T-bet:Eomes expression could predict the functional profile of virus-specific T-cells in several viruses [56]. This study described that depending on differences in viral persistence, virus-specific CD8$^+$ T-cells with a similar phenotype had a different T-bet:Eomes ratio, suggesting that beyond phenotypic differences, the balance in T-bet:Eomes is predictive for differences in T cell function. For the first time the eomes/T-bet expression after yellow fever vaccination was longitudinally evaluated. At the late time point, 180 days after vaccination, the T-bet:Eomes balance shifted in favor of Eomes over T-bet in all YF tetramer-specific CD8$^+$ T-cell subsets. The data suggest that following vaccination, YF-specific cells may potentially be maintained for prolonged periods of time. From the perspective that virus-specific T-cells are maintained through homeostatic proliferation as shown in mice studies [57, 58], the expression of Ki67 as marker for active cellular replication was analyzed; and we observed at day 180 after vaccination that at least in a subgroup of individuals YF-specific cells were Ki67 positive. The observation of proliferating Ki-67 positive cells further support the capacity of self-renewal and potential long term maintenance. An unanswered question is which factors contribute to this proliferation. One possibility is that the presence of YF-antigen may contribute to continuing proliferation. However, continued presence of antigen after YF fever vaccination is debatable, with 1 study showing that no YF-antigen could be detected 11 days after vaccination [59].

The YF-tetramer$^+$CD8$^+$ T-cell pool at day 180 after vaccination showed a heterogeneous distribution: in addition to late differentiated cells, a significant fraction of YF tetramer positive CD8$^+$ T-cells had characteristics of naive cells (CD45RA$^+$ and CD27$^+$). This population further resembles naive cells with regard to high expression of CD28, and low granzyme B and K expression, but has differentiated further [48]. The level of expression of both transcription factors is higher than would have been the case in naive cells (but comparable to early-differentiated cells), suggesting that these cells have probably differentiated further than naive cells. The added value of measuring transcription factors

is illustrated by the fact that we found additional clues about the differentiation process of T-cells.

In our study, we found that booster vaccination neither increased the frequency nor the phenotypic distribution of YF-specific cells compared to primary vaccination. It is known that the magnitude of the T-cell memory response is dependent on the amount of antigen [60]. Non-replicating vaccines do not reach sufficient antigen content and booster doses are required to result in an increased pool of memory cells [61]. In the case of live attenuated vaccinations, booster vaccinations result in a limited increase in the pool of memory CD8+ T-cells and B-cells [61], probably due to rapid neutralization of the antigen in secondary challenge and because of the optimal antigen load upon primary vaccination. In line with these studies, we did not find further induction of the frequency of YF-tetramer+CD8+ T-cells upon booster vaccination.

Taken together, these data lead to the assumption that once a YF-specific CD8+ memory T-cell pool is induced upon vaccination, a booster does not result in higher frequencies or changes in subsets as reflection of changes in function.

The second arm of immunity important in protection after vaccination are neutralizing antibodies. The recent review by Gotuzzo et al summarized previous studies on the duration of antibody presence [3]: one study showed that 80.6% (N=83/103) of veterans presumably vaccinated 30-35 years ago were seropositive [10] and Niedrig and colleagues found 74.5% (N=38/51) volunteers seropositive 11-38 years after vaccination [9]. Coulange and colleagues even showed the presence of antibodies in 1 individual 60 years after vaccination [62]. We found that YFV-neutralizing antibodies were measurable up to 40 years after vaccination, which complements the findings from previous studies. Similar to previous studies [9, 62, 63], we found a correlation between the antibody titers and time since vaccination.

In summary, after single YF vaccination, a clear population of YF-tetramer positive late-differentiated and early-differentiated memory CD8+ T-cells is maintained for at least 18 years. This YF-tetramer+CD8+ T-cell population has the properties of memory cells with a direct cytotoxic potential and a transcriptional profile compatible with long-term maintenance. Boosting of these cells does not lead to further induction of their frequencies and also not to a boosting of the YF-specific humoral immune response. These data provide an additional rationale for the non-necessity for booster vaccination and thereby favoring, fast-tracking the alleviation of booster vaccination requirements in clinical practice.

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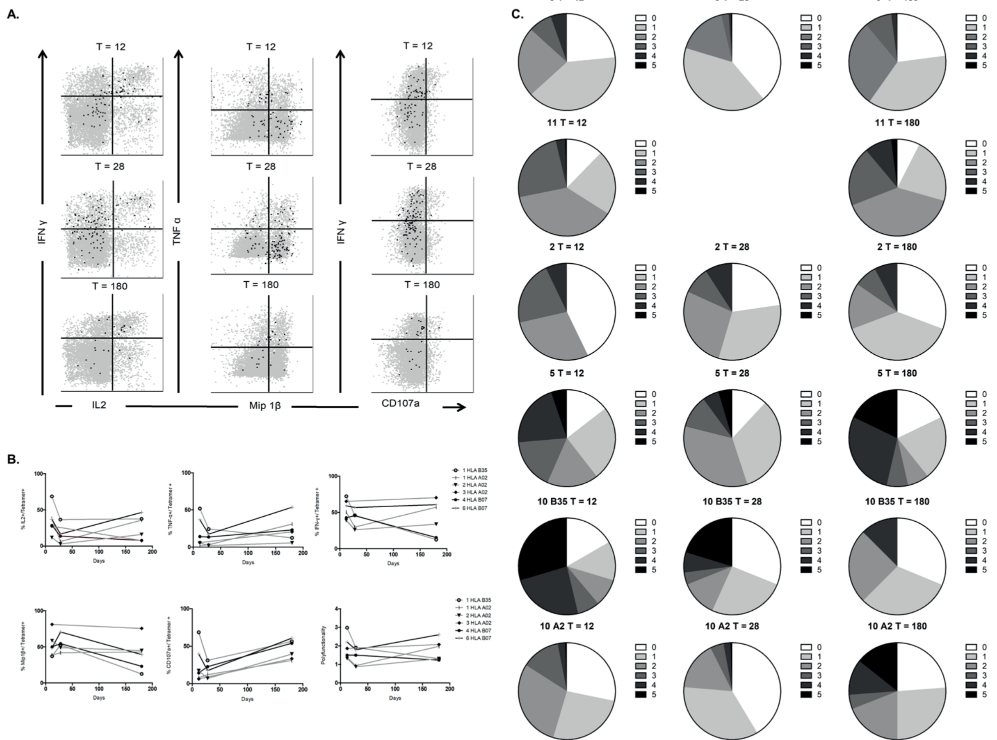
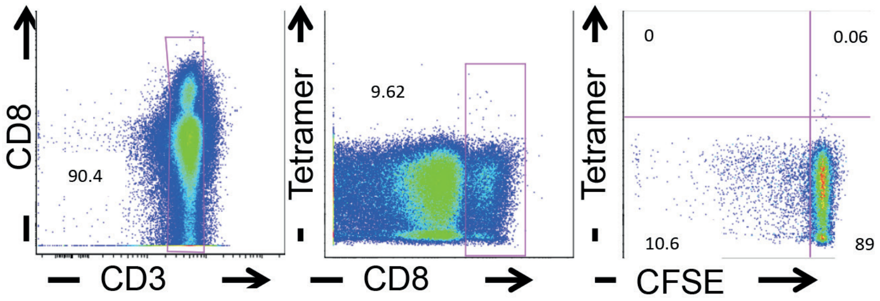
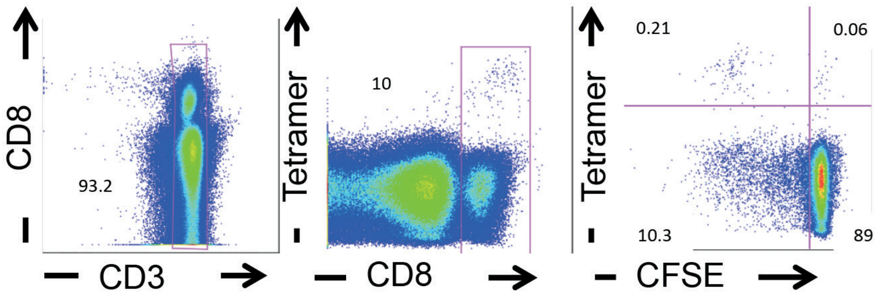


Supplemental Figure 1: Correlation between YF-Antibody titers in 99 individuals that received a single vaccination and time since vaccination. The y-axis shows the time since vaccination and the x-axis shows the YF-serum antibody titer. The correlation between serum titer and time since vaccination was calculated with Spearman's Rank Correlation coefficient. As a reference, the red line depicts the YF antibody serum level threshold of protection (0.5 IU/mL)





Supplemental Figure 2: Representative FACS plots showing proliferation of YF-tetramer positive CD8+ T cells after labeling with carboxyfluorescein succinimidyl ester (CFSE) and 9 days of culturing in the presence of A: IL-2 only B: IL-2 and YF-peptide.

Supplemental Table 2: Demographic details of 13 single vaccinated and 7 boosted individuals of whom PMBC's were collected.

Sex, m (%)	4 (31.0)	1 (14.3)
Age y		
mean (SD)	43.0 (14.0)	54 (12.9)
median [IQR]	37.0 [26-46]	55 [52-62]
Years since last vaccination		
mean (SD)	10.3 (7.7)	9.3 (12.5)
median [IQR]	10.0 [3.0-13.3]	6.0 [2.5-7.5]
Years between 1st and 2nd vaccination (n=3)		
mean (SD)		14.5 (7.6)
median [IQR]		11 [10.5-16]
Years between 2nd and 3rd vaccination (N=4)		
mean (SD)		15.3 (5.0)
median [IQR]		15 [13-18]

Supplemental Table 3: Demographic details of 99 participants vaccinated 11-40 years ago of whom serum was collected. GMT: geometric mean titer.

Sex, m (%)	35, 35.3% (m)
Age (y)	
mean (SD)	49.5 (12.5)
median [IQR]	49 [42.0-56.0]
Time since Vaccination (y)	
mean (SD)	18.2 (6.94)
median [IQR, range]	16.0 [13.0-21.5, 11-40]
GMT Median (range), Mean (SD)	
11-20 years after vaccination (n=73)	5.20, [0.00-71.60], 9.81 (13.0)
21-30 years after vaccination (n=20)	5.10, [0.00-24.90], 6.37 (6.9)
31-40 years after vaccination (n=6)	5.00, [2.30-83.90], 17.63 (32.5)

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Chapter 6

Comparison of the immunogenicity of Dukoral oral cholera vaccine between renal transplant recipients on either a calcineurin inhibitor or mycophenolate

A controlled trial

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6.1 Abstract

Background

The evidence for recommendations regarding vaccination in solid organ transplant recipients is sparse. There is little data comparing vaccine responses between groups on different immunosuppressive drugs. This study was conducted to evaluate the antibody response to Dukoral® oral cholera vaccine in renal transplant recipients (RTR).

Methods

In a single-center non-randomized controlled clinical trial, healthy volunteers (n=21) and renal transplant recipients (n=30) were vaccinated with the oral whole cell/recombinant B subunit cholera vaccine Dukoral® (Valneva Inc., Vienna, Austria). The RTR were stratified according to their maintenance immunosuppressive therapy: either prednisone and a calcineurin inhibitor (cyclosporine A or tacrolimus; P/CNI group; n= 15) or prednisone and mycophenolate (P/MMF group; n=15). All volunteers ingested Dukoral® at baseline and at day 14. Serum samples were drawn at day 0 and day 21. The primary outcome was seroconversion, defined as either a 3-fold IgA serum titer increase in anti-cholera toxin B antibodies and/or a 4-fold rise in the serum vibriocidal titer.

Results

Follow-up was complete. Seroconversion after vaccination was 57% (standard error, SE 9%) in RTR and 81% (SE 9%) in healthy controls (Relative Risk, RR 0.70; 95% CI 0.48-1.02). When stratified according to maintenance immunosuppression, the seroconversion rate was 67% (SE 12%) in the P/CNI group (RR compared with controls 0.82; 95% CI 0.55-1.25) and 47% (SE 13%) in the P/MMF group (RR compared with controls 0.58; 95% CI 0.32-1.03).

Conclusion

Adverse events were mild to moderate and transient. The response to Dukoral was weaker and the seroconversion rate was lower in renal transplant recipients than in healthy controls. In particular, those using mycophenolate had a poor response. Nevertheless, more than half of the transplant recipients seroconverted. Therefore oral vaccines should not be discarded as a potential tool for protection of solid organ transplant recipients.

6.2 Background

After kidney transplantation, immunosuppressive drugs are administered to prevent rejection, delicately balancing improved allograft survival with infectious complications [1]. In solid organ transplantation the standard of care is to administer calcineurin inhibitors (CNI) and mycophenolate. CNI inhibit the intracellular enzyme calcineurin, which plays an important role in transducing the signal from the T-cell receptor to the nucleus to allow transcription of genes encoding for cytokines including IL-2 and the expression of CD40 ligand. Therefore, CNI impair T-cell function including T-cell help to activated B-cells. Mycophenolate interferes with DNA synthesis and is cytotoxic to rapidly dividing cells, such as activated T- and B-lymphocytes. Both drugs severely inhibit the primary and secondary immune response [2]. When using these drugs, the capacity to mount a primary immune response to infection or vaccination is suppressed in a way that is hard to predict in individual circumstances, leading to a variety of reported response rates to different vaccines. There is limited data on the response rate to the majority of vaccines. Results of studies on seroconversion in solid organ transplant recipients after vaccination have been summarized by Eckerle et al. [4].

While guidelines for vaccination of solid organ transplant recipients do exist (such as stated in Kidney Disease: Improving Global Outcomes: KDIGO) [5] these only address a limited number of vaccines and additionally suffer from insufficient adherence in clinical practice, possibly due to uncertainty of transplant doctors regarding the immunogenicity of vaccines under immune suppression [6]. There is virtually no data on responses to oral vaccines [7], such as to the oral cholera vaccine [8] in solid organ transplant recipients. There is some data on the immunogenicity of the oral cholera vaccine in another immunocompromised group: HIV infected Haitian adults[9]. This study showed that 74% of HIV positive subjects seroconverted to the Inaba strain, compared with 91% of the healthy adults. Those with the lowest $CD4^{+}$ count had a poorer response.

Certain groups of travelers, such as renal transplant recipients (RTR) are more vulnerable to the adverse consequences of travelers' diarrhea. Dehydration can induce kidney injury. Therefore, in some countries, Dukoral®, an oral cholera vaccine, is prescribed to immunocompromised travelers to prevent traveler's diarrhea, based on the notion that the immune response to cholera toxin B may provide protection against travelers' diarrhea caused by the heat-labile toxin of enterotoxigenic E. Coli, with which it shares structural and antigenic similarities [10, 11]. This practice is not supported by the evidence, as is summarized in a Cochrane review [12]. Nevertheless, it raises an interesting

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question: To what extent is the response to oral immunization affected by immunosuppressants?

Vaccination of solid organ transplant recipients offers the possibility to study the effect of different immunosuppressive drugs on the ability to mount an immune response. To this end, we performed a non-randomized controlled clinical trial with Dukoral® oral cholera vaccine in RTR on maintenance immunosuppressive therapy with either a calcineurin inhibitor or mycophenolic acid.

6.3 Methods

Study design

This was a single-center non-randomized controlled clinical trial conducted between March 2010 and November 2014 at Leiden University Medical Center (LUMC) in the Netherlands. The primary objective was to evaluate the immunogenicity of 2 doses of the oral whole cell/recombinant B subunit cholera vaccine Dukoral® in RTR (n=21), 21 days after vaccination. The RTR were stratified into two groups according to their maintenance immunosuppressive therapy, either prednisone and a calcineurin inhibitor (cyclosporine A or tacrolimus) (P/CNI group) (n=15) or prednisone and mycophenolic acid (P/MMF group) (n=15). Secondary objectives were to compare the immunogenicity of Dukoral® between RTR and healthy controls and to compare the immunogenicity between the P/CNI group and the P/MMF group. We originally intended to include a third group of RTR: those on prednisone and an mTOR-inhibitor (p/mTORi). However, inclusion into this category was unsuccessful due to sparsity of RTR with p/mTORi in our source population.

RTR were selected from the registry of the department of Nephrology at LUMC. All patients that met the inclusion criteria during the screening period were invited to participate by letter. Concurrently, siblings and partners of the transplant recipients were invited to participate as healthy controls. Adult RTR, with stable renal function and on a stable immunosuppressive regimen consisting of P/CNI or P/MMF were eligible. Exclusion criteria included: a history of auto-immune disease, prior cholera vaccination or infection, use of immunosuppressive medication other than a CNI or MMF, recent treatment with blood products (< 3 months) and recent treatment for graft rejection (< 12 months). Recent episodes of travelers' diarrhea (< 6 months ago) were recorded in the CRF, but were not an exclusion criterium.

The primary immunogenicity endpoint was seroconversion among all RTR. There is no established immunological correlate of protection. The assays were performed by Crucell (Crucell Holland BV).

In accordance with their specifications, seroconversion was defined as a ≥3-fold rise in serum anti-CTB IgA antibodies and/or a ≥4-fold rise in serum vibriocidal antibodies.

Vaccine and procedures

Dukoral® (The pharmaceutical company that produced the vaccine at the time of the study was Crucell Holland BV. The licence is currently held by Valneva Inc., Lyon, France), a licensed oral cholera vaccine consisting of killed whole cell monovalent Vibrio cholerae (serogroup O1, Inaba and Ogawa strain) combined with recombinant cholera toxin subunit B, was administered to all subjects. Subjects received the first dose upon inclusion, according to the instructions of the manufacturer (day 0), and were instructed how to store and self-administer the 2nd dose at home, 2 weeks after the first dose. Administration of the 2nd dose was verified by telephone. Subjects kept a diary of adverse events for 4 days after each dose. Subjects were invited back to the outpatient clinic for a second and final visit, 1 week after the 2nd dose (day 21), at which time the diary was collected. Blood samples were drawn upon inclusion (day 0) and at the final visit (day 21). Samples were centrifuged and serum was stored at -20 °C.

Immunogenicity assays

Anti-CTB serum IgA ELISA: rCTB peptide (Crucell) was coated (2h at room temperature) to high binding microtiter plates (Immunon 2HB, NUNC, USA) at a concentration of 0.5 µg/mL in phosphate buffered saline (PBS). Upon coating, plates were blocked with 1% Casein blocking buffer (Novagen, Merck Millipore, Germany) to reduce the background. Heat-inactivated (30 min at 56°C) samples, a reference sample and internal controls were diluted in blocking buffer and applied to the rCTB coated plate (room temperature). After 2 hours of incubation, and washing with phosphate buffered saline containing 0,05% Tween 20, anti-human IgA HRP-labeled antibody (24 ng/mL, Jackson ImmunoResearch Europe Ltd, UK) was added for 1 hour, followed by tetramethylbenzidine substrate (Sureblue, KPL Inc., USA) for detection. After 10 minutes a 1 M H2SO4 stop solution was used to stop the colorimetric reaction. The optical density (OD) was measured at 450 nm, using a microplate spectrophotometer (PowerWave 340, Bio-Tek, USA). A stored serum sample with an unequivocal reproducible response was used as the reference standard in this assay. The reference value, in relative ELISA units (EU/mL), for this sample was based on the geometric mean 50% infliction point as determined in eight subsequent assay runs. This reference curve was tested at seven 2-fold dilutions from 1/80 to 1/5120 in each assay and a four-parameter logistic (4PL) curve fit was applied. The CTB ELISA titer of individual samples was determined by correlating a single dilution of the

sample in the reference curve. Titers below 0.159 were considered as 0.079 for analysis. The cut-off for seroconversion was established as a 3-fold increase of post- vs. pre-vaccination individual titers.

Vibriocidal assay: V. cholerae O1 Inaba El tor (strain T19479) working freezer stocks were harvested by centrifugation (2 min, 15000 rcf) and washed twice in saline to remove the storage medium (LB medium with 15% glycerol) and diluted to a final OD600 of 0.220 ± 0.01. An assay reaction mixture was prepared by further dilution of the bacteria to 1/80 in 0.85% saline (Fluka, Sigma-Aldrich, USA) solution, supplemented with 6.7% guinea pig complement (Calbiochem, USA). All wells of a 96-well microtiter plate (Immulon 2B, Nunc, USA) were filled with 25 µL of sample. Heat-inactivated (30 min, 56°C) samples and internal controls were serially diluted two-fold in 0.85% saline buffer, starting from 1:5 until reaching 1:1280 dilution. An equal volume (i.e. 25 µL) of the reaction mixture was added to the serially diluted serum samples and allowed to incubate at 37 °C for 1 h and 350 rpm. After 1 hour, 150 µL of fresh brain heart infusion (BHI) media (Prolab, Brazil) was added to each well, and the plates were incubated for an additional 2.5 hours at 37°C. The bacterial turbidity within each well was read at 630 nm with a microtiter-plate reader (PowerWave 340, Bio-Tek, USA) and a four-parameter logistic (4PL) curve fit was applied. The vibriocidal antibody titer was defined as the sample curve's 50% infliction point (i.e. the EC50). Titers below 5 were considered as 2.5 for analysis. The cut-off for seroconversion was defined as a ≥4-fold rise in serum vibriocidal antibodies.

Both assays were performed by Crucell Holland B.V. (currently part of Janssen Pharmaceuticals) after completion of the study. Laboratory workers of Crucell were blinded to the study groups.

Statistical analyses

No formal sample size calculation was performed. Geigy scientific tables were used to estimate the confidence intervals when a total of n=20 renal transplant patients per study arm would be included. This was judged to yield sufficient difference between the groups based on the expected outcome. Because of slow recruitment the study was terminated when 15 RTR were included in the P/CNI and P/MMF study arm.

Statistical analyses were performed using SPSS Statistics version 20.0.0.1 (IBM Corp., USA) and Excel version 2010 (Microsoft Corp., USA).

Ethics statement

All participants provided informed consent. The study was approved by the Medical Ethics

Committee of Leiden University Medical Center and registered in clinicaltrials.gov under NCT01109914.

6.4 Results

Participants

Participant characteristics are summarized in table 1. In total 51 subjects were enrolled and vaccinated, consisting of 30 renal transplant recipients (15 in the P/CNI arm and 15 in the P/MMF arm), and 21 healthy controls (8 patient partners and 13 non-related subjects). All subjects had a negative history for cholera infection or vaccination, and follow-up was complete. Three subjects experienced travelers' diarrhea in the 6 months before inclusion: 2 healthy controls and 1 in the P/MMF arm. None of the subjects had ever been diagnosed with cholera. The dosage of the immunosuppressants was in the therapeutic range, as reflected by serum monitoring. Patients on MMF were slightly older and were more likely to have received past treatment for allograft rejection. The prednisone dosage was slightly higher in the MMF group, in accordance with the protocol for dosage of immunosuppressants after transplantation.

Immunogenicity

At 21 days after vaccination, the overall seroconversion rate was 57% (SE 9%) in renal transplant

Table 1: Characteristics and demographics of the study arms

Variable	HC (n=21)	RTR (n=30)	P/CNI (n=15)	P/MMF (n=15)
Male gender, n (%)	11 (52)	25 (83)	12 (80)	13 (87)
Age, median years (IQR)	49 (24)	60 (23)	56 (26)	62 (15)
Primary kidney disease, n (%)				
Glomerulonephritis	-	9 (30)	3 (20)	6 (40)
Diabetes mellitus	-	1 (3)	0	1 (7)
Hypertension/ischemic	-	4 (13)	2 (13)	2 (13)
ADPCKD	-	6 (20)	2 (13)	4 (27)
Reflux nephropathy	-	3 (10)	3 (20)	0
Other	-	3 (10)	2 (13)	1 (7)
Unknown	-	4 (13)	3 (20)	1 (7)

Table 1: Characteristics and demographics of the study arms - Continued

Variable	HC (n=21)	RTR (n=30)	P/CNI (n=15)	P/MMF (n=15)
Transplant details*				
Years after transplant, mean yrs (range)	-	9.3 (1.8-17.3)	9.3 (2.0-16.6)	9.4 (1.8-17.3)
Heart beating donor, n (%)	-	13 (43)	5 (33)	8 (53)
Non-heart beating donor, n (%)	-	17 (57)	10 (67)	7 (47)
Related donor, n (%)	-	7 (23)	4 (27)	3 (20)
Unrelated donor, n (%)	-	23 (76)	11 (73)	12 (80)
Repeat transplant, n (%)	-	1 (3)	1 (7)	0
Anti-rejection therapy, n (%)	-	5 (17)	1 (7)	4 (27)
Immunosupression				
Prednisone dose, mg, mean (range)	-	7.6 (5-10)	6.7 (5-10)	8.6 (5-10)
Ciclosporin dose, mg, mean (range)	-	-	182 (150-225)	-
Ciclosporine trough level, ug/L, median (IQR)	-	-	103.5 (50.25)	-
Tacrolimus dose, mg, mean (range)	-	-	4.9 (1.5-10)	-
Tacrolimus trough level, ug/L, median (IQR)	-	-	7.8 (4.5)	-
Mycophenolate dose, mg, mean (range)	-	-	-	1929 (1440-2500)
Mycophenolate AUC, mg*h/L, mean (range)	-	-	-	64.5 (44-94)
Laboratory measurements				
Serum creatinin, umol/L, mean (range)	-	117 (73-184)	128 (83-181)	106 (73-184)
GFR Cockroft, mL/min, mean (range)	-	-	63 (30-103)	80 (46-110)
eGFR, mL/min/1.73 m^2, mean (range)	-	-	47 (33-58)	56 (32-81)
Hemoglobin, mmol/L, mean (range)	-	-	8.2 (6.5-10.2)	8.6 (7-10)

Table 1: Characteristics and demographics of the study arms - Continued

Variable	HC (n=21)	RTR (n=30)	P/CNI (n=15)	P/MMF (n=15)
Blood group				
A, n (%)	-	10 (33)	5 (33)	5 (33)
B, n (%)	-	6 (20)	3 (20)	3 (20)
O, n (%)	-	12 (40)	5 (33)	7 (47)
AB, n (%)	-	1 (3)	1 (7)	0

recipients and 81% (SE 9%) in healthy controls (RR for seroconversion in RTR versus controls 0.70; 95% CI 0.48-1.02) (Table 2). When stratified according to maintenance immunosuppression therapy, the seroconversion rates were 67% (SE 12%) in the P/CNI group (RR for seroconversion in the P/CNI group versus controls: 0.82; 95% CI 0.55-1.25) and 47% (SE 13%) in the P/MMF group (RR for seroconversion in the P/MMF group versus controls 0.58; 95% CI 0.32-1.03).

Table 2: Serologic response to Dukoral vaccine in RTRs

	Healthy controls (n=21)	RTR (n=30)	P/CNI (n=15)	P/MMF (n=15)
Combined seroconversion, % (SE; n)	81 (9%, 17)	57 (9%, 17)	67 (12%, 10)	47 (13%, 7)
Risk ratio for seroconversion (95% CI)	1.0 (ref)	0.70 (0.48-1.02)	0.82 (0.55-1.25)	0.58 (0.32-1.03)
Anti-CTB IgA titre, geometric mean (95% CI)				
Baseline	0.3 (0.2-0.5)	0.2 (0.1-0.3)	0.30 (0.2-0.5)	0.2 (0.1-0.3)
Post-vaccination	4.5 (2.3-8.7)	1.0* (0.5-1.9)	2.1 (0.8-5.1)	0.4** (0.2-1.0)
Mean Fold Increase	13.4 (6.7-26.7)	4.3 (2.5-7.6)	6.9 (2.7-17.7)	2.7*** (1.6-4.7)
Vibriocidal titre, geometric mean (95% CI)				
Baseline	26.5 (9.7-72.5)	47.2 (20.0-111.6)	54.8 (15.2-197.4)	40.6 (12.4-133.4)
Post-vaccination	78.0 (26.5-229.8)	86.8 (36.5-206.3)	101.5 (26.9-383.0)	74.3 (23.5-235.0)
Mean Fold Increase	2.9 (1.8-4.7)	1.8 (1.1-3.1)	1.9 (0.9-3.7)	1.8 (0.8-3.9)

CTB: Cholera Toxin B-subunit, CI: Confidence interval (normal distribution)
* Significantly different from control (Mann-Whitney U=166, p=0.004)
** Significantly different from control (Mann-Whitney U=50, p=0.001)
*** Significantly different from control (Mann-Whitney U=171, p=0.006)

Figure 1: Reverse cumulative distribution curves for the anti-cholera toxin B (anti-CTB) titers (A) and the vibriocidal titers (B), indicated in blue for controls, red for renal transplant recipients (RTR) on calcineurin inhibitor + prednisone (P/CNI) and green for RTR on mycophenolate and prednisone (P/MMF). Please note the ordering of anti-CTB responses where the control group has the best response, followed by the P/CNI group and lastly the P/MMF group. Also note the lack of differentiation between vibriocidal responses.

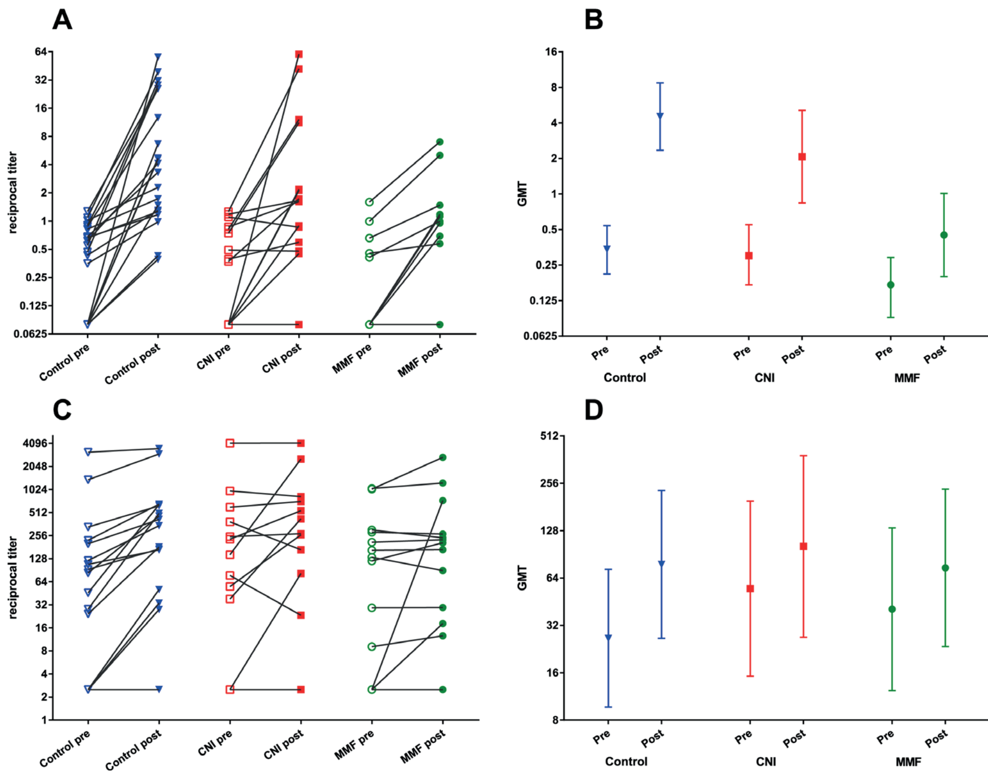

Figure 2: Individual titer plots for anti-CTB titers (A) and for vibriocidal titers (B), and their respective geometric mean titers (B and D). Please note the differential response seen in the anti-CTB titers but not in the vibriocidal titers.

Anti-CTB response: The geometric mean fold increase (GMFI) for anti-CTB IgA titers was 4.3 (95% CI: 2.4-7.8) for RTR and 13.4 (95% CI: 6.4-28) for healthy controls. The GMFI was significantly lower in the P/MMF group than in healthy controls (2.73 vs 13.4; p=0.006). Vibriocidal response: Vibriocidal geometric mean titers (GMT) showed little difference between the study groups, due to high variability in baseline titers between individuals. The GMFI for vibriocidal titers was 1.8 (95% CI: 1.1-3.1) in RTR and 3.0 (95% CI: 1.8-4.8) in healthy controls. For both assays, the reverse cumulative distribution curves are provided in figure 1 and the individual titer plots and geometric mean titers in figure 2.

In total, 34 subjects seroconverted, satisfying either the anti-CTB response criterium (n=21) or the vibriocidal response criterium (n=3) or both (n=10). The strength of the anti-CTB and the vibriocidal response did not correlate with each other or with patients' age, hemoglobin level, serum creatinine,

immunosuppressant concentrations, cumulative prednisone dose or time since transplantation (data not shown).

Table 3: Adverse events after vaccination

	RTR (n=30)		HC (n=21)		Total (n=51)	
	dose 1	dose 2	dose 1	dose 2	dose 1	dose 2
Percentage of subjects with any AE, % (n)	13 (4)	20 (6)	52* (11)	38 (8)	29 (15)	27 (14)
Frequency of various AEs, n:						
bloating	2	-	2	1	4	1
flatulence	1	1	2	2	3	3
decreased appetite	-	-	1	1	1	1
urge to defecate without production	-	-	1	-	1	-
fatigue	-	1	1	1	1	2
excess bowel noises	1	2	3	-	4	2
acid reflux	-	-	1	-	1	-
abdominal pain	1	-	1	1	2	1
nausea	1	1	-	-	1	1
myalgia	-	1	-	-	-	1
general malaise	-	2	-	-	-	2
headache	-	-	1	-	1	-
decreased visual acuity	-	-	1	-	1	-
sleeplessness	-	-	1	1	1	1
dizziness	-	1	1	-	1	1
excess micturition / fluid retention	-	-	1	-	1	-
increased menstrual discomfort	1	-	-	-	1	-

* significant difference with RTRs after dose 1, Fisher's exact test =0.004

Vaccine safety

There were no serious adverse events. Adverse events were mild to moderate and transient in all subjects. Healthy controls suffered more varied adverse events than RTR after the first vaccine dose (p=0.004) but not after the second dose, adding up to a higher total amount of AEs in healthy controls. In total, 15 subjects (29%) reported any adverse event after the first vaccine dose. In 14 subjects this was considered related to vaccination. After the second dose, 14 subjects (28%) reported any adverse event, which in 9 subjects was considered to be related to vaccination (Table 3).

6.5 Discussion

This is the first trial to study the immunogenicity of oral cholera vaccine in solid organ transplant recipients. Seroconversion was 57% in renal transplant recipients and 81% in healthy controls. Vaccine responses were highest in healthy controls, followed by the P/CNI group, and lowest in the P/MMF group. Anti-toxin serum IgA responses were significantly lower in RTRs and lowest in the P/MMF group. The vibriocidal antibody response showed no significant differences between groups due to large variability in the baseline titers and responses. Our findings are in line with several other studies, which show that vaccine responses are lowest in patients on mycophenolic acid, higher in patients on calcineurin inhibitors and highest in healthy controls [3, 13, 14] . However for the mucosal vaccine Dukoral we had expected an even lower response in the RTR groups, based on the relatively low seroconversion to oral cholera vaccines even in healthy vaccinees [15]. The difference in seroconversion rates between RTR and HC was in line with the result of a study in HIV infected adults [9].

Significantly fewer adverse events (AEs) were reported by RTRs than by the controls, but only after the first dose. This may be due to the higher tolerance for physical discomfort developed by RTRs during the course of their previous illness. An immunological etiology seems less likely, since the difference occurred only after the first dose and within 4 days of the dose. The frequency of observed AEs were all in the range of expected side effects after oral cholera vaccination [16, 17] .

The results from our trial demonstrate that oral vaccines can be useful as a tool in the protection of solid organ transplant recipients and should not be dismissed beforehand.

Strengths and weaknesses

The main strength of this study is its prospective controlled design, in which only RTRs on dual therapy (prednisone and 1 other immunosuppressive agent) were included, thus enabling a clear comparison of the effect of different immunosuppressive agents on the response to vaccination. Second, there was no loss to follow-up and the trial was performed in a single transplant center which contributed to a high quality of data regarding the patient characteristics. Furthermore, the study used two assays to assess immunogenicity. Both were performed by the company that produces the vaccine in accordance with strict quality criteria. Finally, variation was minimized by limiting the number of vaccine batches to the absolute minimum achievable, since variation in cholera vaccine trials is at least partly due to the fact there is currently no in vitro test to evaluate and compare the potencies of different vaccine lots [18] .

There are two main limitations. First, the study is relatively small and did not achieve the intended sample size. Nevertheless, the standard errors for the proportions achieving seroconversion are within acceptable limits, allowing between-group comparisons and interpretation of the results. Second, the trial lacks a third arm to assess responses in RTRs using sirolimus or everolimus, the inhibitors of the mammalian target of rapamycin (mTOR). This would have been of particular interest, as there is evidence that immunological responses remain relatively intact in subjects using mTOR inhibitors [3, 19, 20]. Furthermore, we did not evaluate the long-term, to assess differences in waning of titers in RTRs and controls, such as is seen following vaccination for hepatitis B [21]. Lastly, there was high variability in vibriocidal assay results. This affects the field of cholera vaccine research in general, which also suffers from a lack of a true immunological correlate of protection [15, 22-24]. We did not collect data on previous travel to cholera endemic countries. In theory this may have influenced baseline titers.

Discussion of the immunogenicity measurements

In our study we used a 3-fold increase in anti-toxin antibody titer as cutoff for seroconversion instead of the more frequently used 2-fold increase. This stricter cutoff was instituted in consultation with the laboratory experts, based on their experience and because a 2-fold cutoff in an ELISA is more likely to introduce false positive results due to inter-assay variability of one dilution step.

We measured seroconversion and not seroprotection as there is no established correlate of protection

against cholera. The correlation between anti-cholera serology and subsequent protection from cholera is an imperfect one [25]. The challenges of finding a correlate of protection are well illustrated by a challenge study with the antigenically similar Escherichia coli heat-labile toxin [26]. Furthermore, the endpoint in this study was seroconversion, which does not reflect the entirety of the immunological response elicited by an oral vaccine. Assays to study the mucosal immune response (fecal antibody responses, antibody secreting cell response to vaccine antigens) were not performed in this study.

The strength of the vibriocidal and anti-CTB IgA seroresponse did not correlate, probably due to the variability in the results from the vibriocidal assay. This variability is not unique to our study [17, 22]. Furthermore, in contrast to larger studies on oral cholera vaccines, we did not find a correlation between patient characteristics and the immune response [13, 14, 27]. This is to be expected in a study such as ours, with a limited sample size.

6.6 Conclusion

In immunocompromised individuals, the response to vaccination differs, based on the type of immunosuppressant. Therefore, trials of vaccine responses in immunocompromised patients should include well defined groups. Based on this study, we conclude that the response to Dukoral was weaker and that the seroconversion rate was lower in renal transplant recipients than in healthy controls. In particular, those using mycophenolic acid had a poor response. Nevertheless, more than half of the transplant recipients seroconverted and only mild transient adverse events were observed. Therefore, oral vaccines should not be discarded as a potential tool for protection of solid organ transplant recipients.

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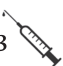
Disclosure

The authors declare that there is no conflict of interest. J&J did not have any part in the design, performance or data analysis of the trial, nor in the writing of this manuscript and did not change



this manuscript prior to publication. No writing assistance was used in the preparation of this manuscript. LV and DS conceived of the research idea, EJ MU and DS executed the trial, EJ analyzed the results under supervision of DS and LV. The manuscript text was authored by EJ, DS and LV and approved by all authors.

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Chapter 7

Summary and discussion

Chapter 2 of this thesis describes a pilot trial that was designed to simplify the vaccination schedule of rabies pre-exposure vaccination (PrEP). Our primary objective was to find the optimal dose for a single-visit schedule to induce sufficient immune memory that can be addressed by revaccination after a future bite exposure. At the time the study was performed the official PrEP schedule consisted of 3 intramuscular vaccine doses requiring 3-4 weeks to complete. Reduction of the number of visits from three to one would very likely increase the uptake of PrEP as both costs and time investment would be significantly less.

A total of 30 healthy volunteers were randomized over 4 study arms, in which they received either 1, 2, or 3 one-fifth fractional intradermal (ID) doses or a full intramuscular dose during a single visit. We included the intramuscular dose because this is the licensed route of administration and does not have the technical difficulties of the intradermal route. Serological follow-up demonstrated rapid waning of the rabies virus neutralizing antibody titers (RVNA): only 27 % of the 28 subjects who initially had seroconverted, still had RVNA titers >0.5 IU/mL one year later. After one year, all subjects received a simulated post-exposure (PEP) vaccination course consisting of two full intramuscular doses two days apart. All 30 subjects demonstrated a robust anamnestic antibody response with RNVA titers >0.5 IU/mL, even the two subjects who did not seroconvert after the primary dose.

Our results confirm previous data from studies exploring shorter pre-exposure vaccination schedules as reviewed by Wieten et al. (1) and Langedijk (2). The results are also in line with data extracted from studies in which RNVA titers were measured after a single dose of a primary series, but where shortening of the vaccination schedule was not the objective of the study. Our results are limited mainly by the small sample size and the lack of a standard treatment arm. Strengths include randomization, laboratory blinding and complete follow-up of all subjects at 12 months. External validity may be negatively impacted by the young age of our volunteers. At older age, the immune system incurs changes that may result in lower seroconversion rates and antibody levels when compared to a younger population. These changes together are termed immunosenescence (3). In the 2-visit intradermal rabies PrEP case series by Mills et al (4) (n=420), seroconversion rates were lower in those over 50 years of age, falling below 90% compared to >95% in the younger age groups. Mansfield et al. (5) found a significantly slower RVNA decline at younger age after the regular 3-visit intramuscular PrEP. Therefore, a single fractional dose of rabies vaccine may not be sufficient in an older population.

The findings of this pilot study formed the basis for a multi-center randomized controlled non-inferiority trial (PREPARE trial) in which the anamnestic neutralizing antibody response to revaccination, six months after a one-fifth fractional intradermal or standard intramuscular dose single visit rabies PrEP is compared to the current standard two visit vaccination regimen (Netherlands Trial Registry; PREPARE NL60550.056.17). This study includes a subgroup of older travelers, to validate single visit rabies PrEP in this age group.

Recently, Soentjens et al. (6) published the results of a study in 303 subjects who received two one-tenth fractional intradermal doses of rabies PrEP in a single visit. Fourteen days after primary vaccination 82.5% of participants seroconverted. This relatively low number is almost certainly explained by the short interval between primary vaccination and serological testing. After 1 year, revaccination with two or four one-tenth fractional intradermal doses resulted in 99.3% seroconversion within one week, thus confirming the presence of robust immunological memory after a single-visit PrEP schedule with two fractional intradermal doses.

Although almost all subjects in our trial seroconverted after primary vaccination, titers decayed fast: after 1 year (before the simulated PEP boosters) only 27 percent of subjects had a titer above the RNVA cut-off of 0.5 IU/mL. In contrast, Mansfield found a seropositivity rate of 86.3% in healthy adults one year after a 3-dose intramuscular vaccination as part of a retrospective case series. A recent systematic review by Stijnis et al. (2) showed that geometric mean titers were lower after an intradermal primary series in comparison with an intramuscular primary series. This difference remained after booster vaccinations. We hypothesize that the slower titer decay after a standard three-visit vaccination schedule is caused by prolonging and expanding the immunological response in the germinal centers by the repeated antigen challenge, leading to the production of higher numbers of plasma cells and more long-lived memory cells. Memory cells that are already present at the time of the second and third dose will also be boosted. In short, the magnitude of the antibody response is related to the amount of antigen exposure and the subsequent 'life span' of the germinal centers, and this may be less after a single-visit vaccination schedule.

Lower antibody titers influence the expected duration of protection after intradermal vaccination, since duration of protection is a function of both the peak antibody titer reached and titer decay. In the context of single visit rabies PrEP, duration of protection should be redefined to include duration of persistence of boostable memory (recently termed boostability in literature (2, 7)), since a protective

antibody titer in the context of rabies is only useful for unnoticed exposures. Currently, it is unknown how long immune memory persists after single visit intradermal rabies vaccination, especially in very low doses such as used here. The duration of protection after single visit vaccination, defined in our study as both the persistence of RVNA > 0.5 IU and as boostability for those who remained or became seronegative, should be investigated through long-term systematic follow-up of vaccinees from current and future trials. This follow-up should consist of 2 parts. Serological follow-up for at least two years, as the decay of RVNA titers is steepest in the first year after primary vaccination (5). Secondly, the boostability should be verified, ideally in a longitudinal study, by means of simulated post-exposure vaccination after 5 years and after 10 years, and longer if possible. This requires a large amount of subjects, since a part of the cohort is lost to follow-up after each evaluation through the act of simulated post-exposure vaccination.

At the time our study was performed the official PrEP schedule consisted of three intramuscular vaccine doses requiring 3-4 weeks to complete. This regimen has since been shortened to two intradermal vaccinations seven days apart (8) to improve accessibility to rabies vaccine. Another approach to improve vaccine accessibility besides dose reduction could be the development of vaccines with different properties according to their intended use, such as described by Ertl et al. (9): pre-exposure and post-exposure prophylaxis are currently achieved by the same vaccine. However, a vaccine used solely for PrEP does not need to have the quality of fast antibody induction, but does need to be cheap. PEP vaccines may benefit from the use of adjuvants to stimulate faster antibody production, but possible cost considerations might make them less suitable for PrEP. These different approaches could be exploited to make rabies vaccination more accessible.

Chapter 3 describes the results of a dose escalation study designed to evaluate the safety and immunogenicity of intradermal (ID) administration of two quadrivalent meningococcal vaccines (MenACWYCRM197 Menveo® and MenACWY-TT Nimenrix ®). It is, to our knowledge, the first time a study on ID administration of a glycoconjugate vaccine was published in literature.

A total of 12 subjects were vaccinated (average 25 years old, range 19–48) in groups of 4 subjects starting at 1/10th of the standard dose, and escalating to 1/5th of a standard dose until seroconversion was observed in at least 3 out of 4 subjects. During the trial immunogenicity was evaluated by means of a multiplex immunoassay (MIA) after each block of subjects. At the end of the trial, antibody titers in all sera were remeasured in a serum bactericidal assay (rSBA, the gold standard) for final analysis.

MenACWY-TT (Nimenrix ®) was evaluated in the 1/10th and 1/5th dose level. MenACWY-CRM197 (Menveo®) became unavailable during the course of the study and was only evaluated for a 1/10th dose.

Intradermal administration of the conjugated MenACWY vaccines was safe: adverse events were mild and there were no serious adverse events. There were no significant differences in geometric mean titer between the vaccines. The tetanus toxoid conjugated vaccine did show a trend towards higher antibody levels compared to the CRM197-conjugated vaccine. Geometric mean titers appeared to decay faster in the MenACWY-CRM197 group. The 1/5th fractional dose of MenACWY-TT appeared to result in higher antibody levels than did the 1/10th dose. This (non-significant) difference in immunogenicity between the vaccines found in our study was not evident from a recent head-to-head vaccine comparison in 64 Korean men who received 1 standard intramuscular dose (10).

Strengths of our study include the randomized design, blinding of the laboratory, and serological evaluation by 2 separate tests. The limited number of subjects is a disadvantage. However, the trial was designed intended to establish safety and the optimal dose for a larger follow-up trial. Young age among the subjects may impact external validity, as do the presence of varying levels of pre-existing anti-meningococcal antibodies despite verbal screening for earlier meningococcal vaccination.

The latter may also be considered an advantage, as it brings the study closer to real life circumstances. It remains unclear if pre-existent immunity influenced the vaccine response. The presence of anti-meningococcal antibodies at the start of the study did not correlate with post-vaccination antibody levels and may have been the result of cross-reactive antibodies to other (enterobacterial) pathogens or commensals such as Moraxella (11). If the observed seroconversion was due to a recall response, those with pre-existing serological immunity should have higher post-vaccination titers, which was not the case.

The MIA failed to show 100% seroconversion for either vaccine, even at the highest dose levels. A finding which was not confirmed when the results of the serum bactericidal assay (SBA) became available at the end of the study. The marked difference in results between the MIA and SBA was unexpected, as the MIA has been shown to correlate well with meningococcal C SBA results (12). However, at the time of the study the MIA was not yet officially calibrated against the SBA for the other serogroups. The difference might also be explained by the nature of the tests: the MIA

quantifies IgG bound to specific polysaccharide chains that are attached to the surface of beads, whereas SBA is a broader, functional assay which measures bacterial killing by all serum components (except complement).

Since the results of the MIA were used for decisions on dose escalation, the trial was amended to include a booster vaccination 4-6 months after primary vaccination. Subjects were revaccinated with an additional intradermal dose according to their original dose level. In the final analysis (using the gold standard: serum bactericidal assay), the post-booster titers were not higher than post-primary vaccination titers in the MenACWY-TT dose levels. In the MenACWY-CRM197 group, the post-booster titers showed a trend towards higher GMT compared to the post-primary vaccination titers. If indeed MenACWY-TT was more immunogenic intradermally, this difference could be due to a 'ceiling effect' in the MenACWY-TT groups.

While revaccination was ultimately superfluous from a seroconversion standpoint, revaccination may contribute to longer duration of protection since significant titer decay was observed for both vaccines. The decline was especially steep in the 1/10th dose levels after 4-6 months, which could be due to early dissolution of the germinal centers, limiting the amount of long-lived plasma cells that were produced. In a study by Ravenhorst et al (13) among adolescents who received an intramuscular TT-conjugated meningococcal C vaccination, antibody titers of all subjects were still above the seroconversion cut-off (rSBA titer >1:8) after 3 years. After 9 years, a single intramuscular menC booster resulted in an antibody half-life of >200 years in the oldest subjects (15 years old) during subsequent follow-up (13). However, it should be noted that titer decline was especially steep in the first year after the booster and leveled off in the subsequent 2 years. So, even though intradermal boosting may extend the duration of protection in a similar way to intramuscular boosting, long-term measurements are required for an accurate estimate of duration of protection since serum IgG decline is not linear over time.

Our trial was originally conceived to precede a larger trial with the ultimate goal to determine the efficacy of dose sparing regimens. If effective, the intradermal administration of a fractional vaccine dose would result in less costs and increased coverage in case of a limited vaccine stockpile. Recently, meningococcal vaccine availability has become problematic, since the introduction of quadrivalent meningococcal conjugate vaccines into national immunization programs has led to vaccine shortages in several high income countries. A fractional dosing schedule may enable a larger

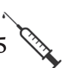
proportion of the population to be vaccinated with the same amount of vaccine.

This issue is not only relevant in high income countries, but in low- and middle income countries as well. For example, multivalent conjugated meningococcal vaccination may be used in Africa's 'meningitis belt' where, after the virtual elimination of serotype A after the successful MenAfriVac campaign, serotypes C and W are currently most prevalent (14). After the MenAfriVac campaign, population health was significantly improved and the economic toll of the disease was reduced (15). It is in these chronically underserved areas that the expected impact of future vaccination campaigns is greatest, based on (conservative) modeling of costs in Burkina Faso (16). The cost-benefit ratio may improve further when taking into account the cost saving effect of fractional dosing strategies such as the one piloted in our trial.

In conclusion, fractional intradermal vaccination with quadrivalent meningococcal conjugate vaccine appears to be safe and sufficiently immunogenic to warrant further study. The 1/5th dose level is recommended for future studies, due to better immunogenicity and also for logistical reasons. An intramuscular control group should be included and titer decay should be measured over several years to evaluate duration of protection and booster necessity for this vaccination modality.

In **Chapter 4**, we retrospectively evaluated the neutralizing antibody response in a series of 15 immunocompromised patients who had inadvertently received a primary yellow fever vaccination while under immunosuppressive therapy. Serum samples were obtained at a median of 33 days after vaccination. At that time antibody responses were determined using an immune fluorescence assay (IFA). We compared these antibody responses with those of 12 matched healthy controls, both in the IFA and the gold standard plaque reduction neutralization test (PRNT).

None of the patients developed a serious adverse event after vaccination. All 15 patients who were vaccinated under immunosuppressive treatment had neutralizing antibody levels above the cutoff in the PRNT. In contrast, only 7 had an IFA titer above the cutoff. In the healthy controls, 10 out of 12 tested positive in the PRNT, whereas none tested positive in the IFA. As a result, the sensitivity of the IFA was only 28% when compared to the gold standard PRNT. The large discrepancy between IFA and PRNT results is difficult to explain. The IFA measures the binding of YF-specific IgG to the surface of infected cells, whereas the PRNT measures antiviral activity in serum of both IgM and IgG. IgM may still contribute to virus neutralization one month after vaccination. This was suggested by the delayed seroconversion of 2 subjects in the IFA (ref: table 1 of chapter 4). Alternatively, the IFA



cut-off of 1:100 may be too high. The possibility of false positives in the PRNT due to cross-reacting antibodies against other flaviviruses is highly unlikely, as an 80% plaque reduction was used as the endpoint in the neutralization assay. Finally, we excluded the possibility of virus neutralization by methotrexate present in the patient's serum by testing the antiviral activity of increasing concentrations of MTX in the PRNT.

The fact that 2 patients unnecessarily had received revaccination based on the negative IFA test result emphasizes the importance of a reliable test. The Euroimmun IFA test as used here was found to be unsuitable for evaluation of the antibodies response to yellow fever vaccine and should not be used.

A prospective observation study in patients on low-dose methotrexate (MTX) was recently published by Bühler et al. (17). It was found that all patients had seroconverted 28 days after primary 17D YF vaccination, confirming both our findings and the majority of available literature, which indicates comparable immunogenicity after YV primary vaccination between patients immunosuppressed by the treatment of immune-mediated inflammatory diseases and healthy controls (18). Neutralizing antibody titers in the Bühler study were slower to develop and were lower at all time points in the patients treated with MTX compared to healthy controls.

We retested our original cohort of 15 patients, now 0 to 18 years after primary vaccination. All patients were still seropositive in the PRNT. Six patients had stopped taking immunosuppressive medication between their primary vaccination and subsequent sampling. In the matched healthy controls, 29 out of 30 were still protected 0 to 22 years after vaccination. These results suggest that the immunosuppressive regimes used in our study had not resulted in a more rapid antibody decay, however the generalizability of this result is limited due to the heterogeneity in immunosuppressive regimens between subjects and the small sample size.

A small series of immunosuppressed patients by Lindsey et al. (19) found that immunocompromised patients were less likely to have a positive PRNT >10 years after vaccination compared to healthy controls: 72 vs 94%. The data did not include immune status at the time of vaccination. De Castro Fereira and colleagues (20) also encountered a difference in seroprotection in a cross-sectional open-label study in a large sample of rheumatoid arthritis patients who were vaccinated while under immunosuppressive treatment. Cellular immunity was also assessed. While those vaccinated between 1 to 5 years ago showed a good seroprotection rate, the patient group treated

with a combination of a conventional and a biological DMARD who were between 5-9 years after vaccination already had a lower seroprotection rate both compared to healthy controls and to those solely on a conventional DMARD in the same timeframe after vaccination. Although individual titers over the years were not measured due to the nature of the study, it would appear that titer decline in those on double therapy starts between 5 to 9 years after initial vaccination, whereas in the healthy population it starts after 10 years. YFV-specific effector memory CD8+ T-cells showed a decline 1-5 years after primary vaccination in the double therapy group whereas in healthy controls and conventional DMARDs the decline starts after 10 years, mirroring the serological results. This T-cell subset was identified as a potential biomarker for use as a correlate of protection by De Castro Ferreira and colleagues (20), and is further referenced in the discussion of chapter 4. The question whether duration of protection is shorter if immunosuppression is started after 17D YF vaccination was addressed by Burkhard et al. (21) who showed no difference in seropositivity between healthy controls and patients who were put on immunosuppressive medication after YF vaccination, with samples taken up to 46 years after vaccination.

In conclusion, immune suppressive therapy with MTX does not appear to hinder seroconversion after 17D yellow fever vaccination, but titers may be lower. The duration of protection in this group is uncertain, but appears unaffected if MTX is started after vaccination. Reports on the effect of other immunosuppressive regimes are conflicting and vary for each vaccine. In clinical practice, seroconversion should be confirmed by PRNT after YF vaccination of an immunocompromised person and thereafter before travel to a high risk area. Collection of additional data of longitudinal vaccine responses and duration of seropositivity should be encouraged to facilitate informed decision making in this vulnerable patient population.

In **Chapter 5** we explored the durability of the immunological response against yellow fever after primary yellow fever vaccination, by determining neutralizing antibody titers and analysis of the phenotype and long-term persistence of yellow fever specific CD8+ T-cells over time.

In a cross-sectional cohort of 99 individuals, 89.9% still had neutralizing antibodies above the threshold after a median interval since vaccination of 16 years (range 11 - 40 years). The majority of these subjects were sampled between 10 and 20 years after vaccination (n=73, of which 66 remained protected). Our study was limited by the small sample size of subjects >20 years post vaccination: 20 subjects were tested between 21 and 30 years after vaccination (of which 17 remained protected), and

6 subjects between 31 to 40 years after vaccination (all protected).

There was a significant inverse correlation between time since vaccination and antibody titer, and between age at vaccination and antibody titer. Lower seroconversion rates in the older population have been described for other vaccines as well (4).

The long-term persistence of seropositivity found in our study is at the higher range reported in literature and supports the WHO policy that a booster vaccination is not necessary in most people. Still, conflicting results and opinions on the need for revaccination remain (22) (23). One explanation for the discrepancy in antibody persistence rates beyond 10 years may be the influence of the immune environment of the vaccinee on vaccine response. Muyanja et al (24) found a lower antibody response in subjects vaccinated in Uganda when compared controls of the same age distribution in Lausanne, Switzerland. They hypothesized that this may be due to an activated immune microenvironment which dampened the 17DD vaccine response in Ugandan cohort. In light of this, the external validity (i.e. generalizability) of our study may be limited to travelers from high-income Western countries, and longevity of the antibody responses should be confirmed in the population in endemic countries.

Neumayer et al. (25) recently proposed revaccination on different grounds: cases without protective antibody titers more than 10 years after vaccination may not only be due to seroreversion but also to primary non-response. The yellow fever booster vaccination (second vaccination) would then be a second chance to respond to the vaccine analogous to the double measles vaccination in many national immunization programs. The known higher seroconversion rate of 17D vaccine (>99%) compared to measles vaccine (~95%) and the comparatively low seroprotection rates found after 10 years in the endemic population contradict this argument. Lindsey et al. noted in their retrospective cohort of Western travelers that all 13 subjects who received with at least 1 booster vaccination were seroprotected regardless of time since vaccination (19). It might only take a single booster dose for seropositivity to last a lifetime. Therefore, a rational middle ground could be to revaccinate (Western) travelers with a single booster and afterwards consider them protected, to be determined based on destination risk, time since vaccination and immune status.

As confirmed by the passive immunization of hamsters (26), neutralizing antibodies confer protective immunity after YF vaccination. However, the role of YF specific $CD8^{+}$ T-cells in the protection against yellow fever remains largely unknown (22). To characterize the YF-specific

T-cell response over time, we also included 6 volunteers for a prospective study. These subjects were vaccinated with 17D YF vaccine, and peripheral blood mononuclear cells (PBMC's) were obtained prior to vaccination and on days 3, 5, 12, 28 and 180 after vaccination. PBMC's were also collected from a subgroup from the retrospective part of the study (n=20).

Up to 180 days after primary vaccination, the phenotype of YF specific CD8+ T-cells did not conform to a typical memory or effector profile. Different viruses are known to give rise to different memory subsets, which might explain these results (27). The cytotoxic potential and polyfunctionality in the expression of cytokines in our study suggest a protective role. We also found that YF specific CD8+ T-cells persisted for at least 18 years after vaccination in our cohort. Their relative frequency was not elevated in those who had received booster vaccinations during their lifetime, suggesting either that these cells do not wane, or that boosting is not possible. In another study by Wieten et al. (28) there was no correlation between the amount of YF specific CD8+ T-cell present in peripheral blood and the serum neutralizing antibody titer.

In the absence of neutralizing antibodies, for example after waning of titers, a yellow fever virus exposure can lead to infection. The incubation period of yellow fever is slightly shorter than the expected time it takes the cellular memory pool to proliferate (29) and definitely shorter than the humoral memory response. In theory, such a cellular memory response in the absence of sufficient neutralizing antibodies might protect from overt disease by aborting a recently established infection where otherwise overt disease might have developed. Immunological memory appears to be lifelong, circulating antibody titers may not be (29).

Costa-Pereira and colleagues (30) attempted to define cellular and cytokine biomarkers for the presence of immunity and memory status after yellow fever vaccination, with the goal of finding correlates of protection. They identified effector memory cytotoxic T-cells (EMCD8) and IL-5-producing T-helper cells (IL-5CD4) as the top biomarkers which should predict the presence of immune memory. This result should be validated in an independent cohort. More than 10 years after vaccination, all memory-related biomarkers decreased considerably in their population, leading the authors to suggest that evaluation of the continued presence of YF-specific immunity is indicated after this time period in those at risk. The latter conclusion is not supported by evidence, since protection is conferred by neutralizing antibodies.

In conclusion, prospective studies are needed into the exact role of cellular immunity in the protection

against yellow fever after vaccination, extending beyond 10 years. Cellular immunological memory might provide sufficient protection to abort a yellow fever infection after antibodies have waned, but a definitive cellular correlate of protection is yet to be identified. Such a finding would confirm the idea of life-long protection in the healthy host, even after antibody waning.

In **Chapter 6**, we compared the antibody responses to oral inactivated cholera vaccine (OCV) Dukoral in renal transplant recipients (RTR) on different dual immunosuppressive regimes (n=15 per group) and healthy controls (n=21) in a non-randomized clinical trial. This enabled us to measure not only the oral vaccine response in this population but also the relative difference in immunosuppressive activity of mycophenolate and calcineurin inhibitors on the primary immune response after mucosal vaccination. This was the first reported trial to study the immunogenicity of oral cholera vaccine in solid organ transplant recipients.

Seroconversion was defined as a combined endpoint of either a 3-fold IgA serum titer increase in anti-cholera toxin B antibodies and/or a 4-fold rise in the serum vibriocidal titer. Seroconversion after vaccination occurred in 57% of RTR and 81% of healthy controls. Especially those using mycophenolate had a poor response with 47% seroconversion as opposed to 67% in subjects on a calcineurin inhibitor. There were no serious adverse events and significantly fewer adverse events in RTR compared to healthy controls. The low performance of the mycophenolate arm is in line with reports in literature for parenteral vaccines such as influenza (31-34). Our results for the healthy controls are also in line with those mentioned in the summary of product characteristics used for market authorization in the EUR region (35).

Strengths include the prospective controlled design, clear separation according to immunosuppressive regime, no loss-to-follow-up and the use of 2 assays to assess vaccine response. Limitations include the relatively small sample size and the large amount of variability especially in the serum vibriocidal titers. This is a known property of the test (35, 36). No long-term follow-up was performed but even in healthy vaccinees there is no real expectation of protection beyond a few years (35).

The best correlate of protection after cholera vaccination is unknown. There is an imperfect correlation between seroconversion and protection, whereby the vaccine protective efficacy is generally higher than the measured seroconversion rates (35). The vibriocidal assay used in our trial showed high variability, which is a known attribute of the assay and affects the entire field of cholera research. Most trials report serum antibodies, but evaluating mucosal immunity by means

of salivary or feces IgA antibodies measurements could add significantly to the body of knowledge regarding mucosal immunity after cholera vaccination and vaccination against mucosal pathogens in the broader sense.

In conclusion, adverse events were mild and transient and significantly less pronounced in RTR than in healthy controls. Seroconversion was lower in renal transplant recipients than in healthy controls, but seroconversion occurred in at least half of RTR. Thus, oral vaccination should not be ruled out in solid organ transplant recipients but vaccine response should always be confirmed.

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Nederlandse samenvatting

Deze Nederlandstalige samenvatting is bedoeld om geïnteresseerden zonder medische of wetenschappelijke achtergrond te informeren over het kader, het doel en de inhoud van dit proefschrift.

Vaccinatie is (naast de verbetering van de algemene hygiëne) de meest effectieve interventie die we kennen in de publieke gezondheidszorg. Waar vaccinatie beschikbaar is heeft het gezorgd voor een enorme daling van ziekte en sterfte in de bevolking. Dit proefschrift richt zich op het verbeteren van de efficiëntie en de beschikbaarheid van vaccinatie. De in dit proefschrift gepresenteerde onderzoeken variëren in onderwerp van het verlagen van de vaccindosis en het veranderen van de toedieningsweg, tot reductie in de toedieningsfrequentie. Tevens omvatten ze effectmaten voor het aantonen van bescherming, de beschermingsduur, en vaccineffectiviteit bij het gebruik van afweeronderdrukkende medicatie. Hieronder volgt per hoofdstuk een overzicht van het kader en de bevindingen van de in het proefschrift opgenomen onderzoeken, tussendoor voorzien van relevante achtergrondinformatie.

In **hoofdstuk 2** van dit proefschrift beschrijven we het onderzoek naar het verkorten van het geldende vaccinatieschema tegen hondsdolheid. Hondsdolheid is een wereldwijd voorkomende ernstige virusinfectie van het zenuwstelsel en de hersenen, die helaas bijna altijd tot de dood leidt zodra de infectie zich gevestigd heeft in het lichaam. Men kan hondsdolheid oplopen door een beet of lik van een besmet dier. De landen waar hondsdolheid het grootste aantal slachtoffers eist liggen in Afrika en Azië. Speciale eiwitten van het afweersysteem (ook wel: immuunsysteem) kunnen bescherming bieden tegen de ziekte door het virus in te kapselen en ter vernietiging aan te bieden. Deze eiwitten worden antilichamen genoemd en spelen een centrale rol bij de bescherming tegen

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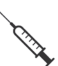


ziekteverwekkers. Om snel voldoende bescherming te bieden tegen deze ernstige ziekte moet het lichaam, liefst voorafgaand aan een blootstelling, al worden getraind om het hondsdolheidsvirus te herkennen en de beschermende antilichamen (zeer) snel te kunnen produceren. Dit wordt bereikt met een vaccin. Bij onderzoek naar de effectiviteit van een vaccin spelen deze antilichamen, gericht tegen het hondsdolheidsvirus, de belangrijkste rol.

We onderzochten de optimale dosis van een bestaand hondsdolheidsvaccin om het afweersysteem van reizigers met slechts 1 bezoek aan de vaccinatiepolikliniek te trainen in het herkennen van het hondsdolheidsvirus. Preventieve vaccinatie tegen hondsdolheid (voorafgaand aan blootstelling door bijtverwonding) wordt PrEP genoemd, een Engelse afkorting die staat voor pre-exposure prophylaxis. Het gebruikelijke PrEP-vaccinatieschema ten tijde van het onderzoek bestond uit 3 doses van het vaccin toegediend in 3-4 weken. Wij wilden de haalbaarheid van een dosissparend en korter vaccinatieschema onderzoeken, omdat veel mensen de tijd niet hebben voorafgaand aan hun reis, omdat vaccinatie tegen hondsdolheid duur is en vanwege regelmatig voorkomende vaccintekorten.

Eén van de reeds bekende strategieën om meer mensen met minder vaccin en/of minder vaccinatiebezoeken te vaccineren, is het gebruikmaken van de eigenschappen van de huid. Normaliter worden de meeste vaccins toegediend in de spier, maar de huid bevat meer immuuncellen dan de spier. Daarom kan men vaak volstaan met het toedienen van een kleinere hoeveelheid vaccin in de huid dan wat men in de spier toedient. Toediening in de huid is echter technisch moeilijker en vereist een hogere vaardigheid en meer training van degene die het vaccin toedient. Toediening in de huid werkt niet voor alle vaccins en ook zijn de huidige flacons waarin de fabrikanten het vaccin leveren niet toegerust op deze manier van vaccineren, waarbij meerdere doses voor meerdere personen uit 1 flacon worden gehaald.

Wij vonden 30 vrijwilligers bereid om deel te nemen aan ons hondsdolheidsvaccinatieonderzoek. Wij verdeelden de proefpersonen willekeurig over 4 verschillende verkorte vaccinatiestrategieën: alle groepen hoefden voor de vaccinatie slechts één keer te komen in plaats van de destijds gebruikelijke 3 keer. Elke groep kreeg het vaccin in een verlaagde dosering. De eerste drie groepen kregen het vaccin in verlaagde dosering in de huid, op 1 plek, 2 plekken of 3 plekken tegelijkertijd toegediend, de laatste groep kreeg één volle dosis in de spier. Het betreft dus zowel minder prikken als een verlaagde dosering per prik (bij de prikken in de huid). Nadien namen wij op vaste tijdstippen bloed af om het

gehalte aan beschermende antilichamen te meten. Na een eerste vaccinatie duurt het doorgaans twee weken tot een maand voordat het lichaam voldoende antilichamen heeft aangemaakt. Eén maand na de vaccinaties met de nieuwe schema's lieten 28 van de 30 deelnemers een afdoende hoeveelheid antilichamen zien. Bij twee deelnemers zagen we onvoldoende beschermende antilichamen: één persoon die de laagste dosis in de huid had ontvangen en één persoon die de gebruikelijke dosis in de spier had gehad.

De hoeveelheid antilichamen in het bloed neemt doorgaans na verloop van tijd af. De snelheid waarmee dit gebeurt verschilt per ziekte, per vaccin, per frequentie van blootstelling en per persoon. Na 1 jaar namen we opnieuw bloed af bij de proefpersonen en was er een flinke afname te zien in het aantal personen dat nog voldoende antilichaam in het bloed had; bij minder dan 1/3e was dit het geval. Vermoedelijk heeft dit te maken met de lagere dosis van het vaccin: er was minder vaccinvirusmateriaal aanwezig om het immuunsysteem te stimuleren, naast het feit dat dit maar eenmalig werd toegediend in plaats van diverse keren, wat de immuunreactie ook langer op gang zou hebben gehouden.

Het verdwijnen van antilichamen in het bloed is bij de bescherming tegen hondsdolheid over het algemeen geen groot probleem, zolang er maar geheugencellen zijn gevormd in het lichaam die snel opnieuw een grote hoeveelheid antilichamen kunnen aanmaken. Het belangrijkste doel van hondsdolheidsvaccinatie is namelijk het aanleggen van deze geheugencellen, klaar om te reageren mocht er in de toekomst onverhoopt een blootstelling aan hondsdolheid plaatsvinden. Na een blootstelling hoort dit proces bij wijze van behandeling zo snel mogelijk op gang gebracht te worden door het opnieuw toedienen van het vaccin (de zogenaamde post-expositieprofylaxe, PEP).

Om de aanwezigheid (of eigenlijk: functie) van deze geheugencellen indirect te testen, gaven we de proefpersonen na één jaar een proefblootstelling, in de vorm gedode hondsdolheidsvirusdeeltjes (opnieuw het vaccin). Binnen één week na deze proefblootstelling was elke proefpersoon beschermd met een robuuste hoeveelheid antilichamen in het bloed, ook de twee personen die aanvankelijk onvoldoende antilichamen in hun bloed hadden. Daarmee is aangetoond dat het op deze manier verkorten van het vaccinatieschema de potentie heeft om meer mensen te beschermen met minder vaccin, waarschijnlijk tegen een lagere tijds- en geldinvestering. Bevestiging van deze resultaten in grotere groepen proefpersonen was nodig om statistische zekerheid te behalen over deze onderzoeksuitkomst: dit is door opvolgende onderzoekers verricht in de vorm van het PREPARE-

onderzoek, waarin ook de over het algemeen lagere effectiviteit van vaccins in de oudere medemens is meegenomen in de onderzoeksopzet.

Voorafgaand aan de bevestiging door het PREPARE-onderzoek bestond overigens al een hoge mate van zekerheid over de juistheid van het behaalde resultaat, omdat in eerdere onderzoeken van anderen hier ook sterke aanwijzingen voor bestonden. Het idee was echter nog nooit met zo'n forse dosisreductie getest als in ons onderzoek. Vervolgonderzoek zal zich voornamelijk moeten richten op het vaststellen hoe lang de functionele geheugencellen na de verkorte hondsdolheidsvaccinatie aanwezig blijven in het lichaam; met andere woorden, hoe lang de reiziger beschermd is na deze verkorte vaccinatie. Hopelijk zal dit levenslang zijn, zoals bij bepaalde andere vaccinaties die in dit proefschrift aan bod komen.

In **hoofdstuk 3** beschrijven wij de onderzoeksresultaten van een gelijkaardig onderzoek als in **hoofdstuk 2**, maar dan gericht op bescherming na vaccinatie tegen meningokokkenziekte met een verlaagde vaccindosis in de huid. Meningokokkenziekte is een ernstige bacteriële infectie die zich kan uiten als hersenvliesontsteking of bloedvergiftiging, beiden vaak met ernstige invaliderende gevolgen. Meningokokkenziekte komt wereldwijd voor, ook in Nederland, wat ertoe heeft geleid dat vaccinatie tegen één variant van deze ziekte sinds 2002 is opgenomen in het Rijksvaccinatieprogramma. Sinds 2018 wordt in het Rijksvaccinatieprogramma gebruik gemaakt van vaccins die bescherming bieden tegen 4 varianten (het zgn. vierwaardige vaccin), dezelfde soort vaccins die in ons onderzoek gebruikt zijn.

Er was nooit eerder onderzocht of vierwaardige meningokokkenvaccins (die bescherming bieden tegen 4 van de meer dan 6 ziekmakende varianten van deze bacterie) effectief en veilig zijn als ze in lagere dosering in de huid worden toegediend. Ons onderzoek richtte zich dan ook op deze vraag, als zogenaamd haalbaarheidsonderzoek. Wij maakten hierbij gebruik van 2 bestaande vierwaardige vaccins (Menveo® en Nimenrix®). Het onderzoek werkte volgens een getrapte dosisescalatiestrategie om met zo min mogelijk proefpersonen de meest geschikte vaccindosis te bepalen om in de toekomst een groter vervolgonderzoek op te zetten. In totaal werden 12 proefpersonen in het onderzoek opgenomen, die willekeurig werden ingedeeld in opeenvolgende blokken van 4 en vervolgens werden gevaccineerd. Alle proefpersonen werden gevaccineerd in de huid. Er werd gestart met 1/10e van de gebruikelijke spierdosis. Als er onvoldoende reactie was in tussentijdse evaluatie van de immuunreactie, werd een tweede blok proefpersonen gevaccineerd met 1/5e van de gebruikelijke

dosis. Eén van de vaccins (Menveo®) was midden in het onderzoek plotseling niet meer beschikbaar, waardoor van dit vaccin alleen de 1/10e dosering in de huid is onderzocht.

In deze kleine groep proefpersonen bleek toediening van deze vaccins in de huid veilig. Op basis van de tussentijdse meting van het gehalte aan bacteriebindende antilichamen besloten wij om iedere deelnemer na 4-6 maanden opnieuw een lage dosis in de huid te geven, in dezelfde hoeveelheid als de betreffende persoon in het eerste deel van het onderzoek had ontvangen. Achteraf, na meting van het gehalte aan bacteriedodende antilichamen, bleek dat ook het toedienen van 1 dosis in de laagste dosering in de huid vermoedelijk al voldoende beschermende antilichamen opwekte. In ieder geval is het toedienen van 2 doseringen met 4-6 maanden tussenpoos effectief in alle dosisniveaus voor alle in het vaccin opgenomen bacterievarianten.

Een factor die de resultaten onnauwkeuriger maakt is de grote variatie in het gehalte aan antilichamen die we maten in de proefpersonen, reeds voorafgaand aan de vaccinatie in dit onderzoek. Mogelijk waren ze ongemerkt al eens blootgesteld aan de bacterie of was er toch sprake van eerdere vaccinatie, hoewel dat als uitsluitingsgrond in het onderzoek was opgenomen. Tegen deze verklaringen pleit dat er geen booster-effect werd waargenomen: er was geen sterke toename van de hoeveelheid antilichamen zoals verwacht wordt als iemand al eerder een afweerreactie tegen een ziekteverwekker heeft gemaakt. Er kan ook sprake zijn van kruisreactiviteit van antilichamen tegen verwante bacteriën die de neus- en keelholte kunnen bewonen. Kruisreactiviteit is een bekend fenomeen bij antilichaamtesten.

In tegenstelling tot bij hondsdolheid, is het bij meningokokkenziekte wél van groot belang dat gevaccineerde personen altijd een voldoende hoog niveau van beschermende antilichamen in hun bloed behouden, omdat de meningokokkenbacterie ongemerkt in de bevolking aanwezig is en men nooit weet wanneer er een blootstelling plaatsvindt. Omdat bekend is dat vaccinatie met een verlaagde dosis in de huid een lagere hoeveelheid antilichamen oplevert dan vaccinatie in de spier, is het bij deze manier van vaccineren tegen deze ziekteverwekker van groot belang om de duur van de bescherming te onderzoeken. De tweede dosis na 4-6 maanden zorgt er voor dat het antilichaamniveau in het bloed langer hoog blijft en mensen langer beschermd blijven.

Het schema zoals door ons onderzocht heeft niet alleen potentiële voordelen in Nederland bij vaccintekort, maar ook in gebieden op de wereld waar meningokokkenziekte, door gebrek aan financiële middelen en publieke gezondheidszorgvoorzieningen, een veel groter probleem is dan

in Nederland. Een van deze gebieden is de zogenaamde 'meningitisgordel' of 'meningitis belt' in Afrika, waar reeds eerder geïmplementeerde tijdelijke vaccinatieprogramma's tot een aantoonbare verbetering van de volksgezondheid en vermindering van de economische impact van de ziekte hebben geleid. In dergelijke omstandigheden is het meeste rendement te verwachten van (zo efficiënt mogelijke) vaccinatiecampagnes.

In **hoofdstuk 4** onderzochten we het effect van vaccinatie tegen gele koorts in een serie van 15 patiënten wiens immuunsysteem door behandeling deels was onderdrukt en die per abuis het levend-verzwakte gelekoortsvaccin ontvingen.

Het immuunsysteem is, zoals gezegd, het deel van het lichaam dat infecties bestrijdt, en is als zodanig essentieel voor de bescherming die ontstaat na vaccinatie. Vaccinatie werkt in het algemeen als een ongevaarlijke 'nepinfectie' om het lichaam te trainen voor het geval er later een echte infectie optreedt. Bij iedere ziekte/infectie zijn er ongewenste bijverschijnselen, zo ook bij het gebruik van vaccins. Deze ongewenste bijverschijnselen zijn vrijwel altijd mild en voorbijgaand van aard, maar in zeldzame gevallen ontstaat een ernstige bijwerking. Het gelekoortsvaccin is een zogenaamd levend verzwakt vaccin, hetgeen betekent dat het ziekmakende virus 'levend' in het vaccin aanwezig is, maar daarbij zodanig verzwakt is dat een normaal werkend immuunsysteem het vaccinvirus goed kan bestrijden. Als het immuunsysteem onderdrukt is, bijvoorbeeld door een behandeling tegen reuma of na een niertransplantatie, bestaat er een grotere kans dat het verzwakte virus wél sterk genoeg is om de afweer van het immuunsysteem te overwinnen. Dit kan leiden tot een dodelijke infectie met het vaccinvirus, vandaar dat mensen met een dergelijk onderdrukt immuunsysteem het gelekoortsvaccin niet mogen ontvangen. Toch gebeurt dat af en toe per abuis, hetgeen een mogelijkheid oplevert om hier onderzoek naar te doen.

Ruim een maand na de abusievelijke vaccinatie werd bij de patiënten bloed afgenomen om het gehalte aan beschermende (neutraliserende) antilichamen te meten. Hierbij werden 2 methoden gebruikt en met elkaar vergeleken: een relatief eenvoudige methode gebaseerd op het zien van oplichtende antilichamen die zich hebben gebonden aan virusdeeltjes op een plaat (immuunfluorescentie-assay, IFA), en de bewerkelijke gouden standaard. Hierbij wordt serum met levend virus gemengd en vervolgens wordt gemeten hoe neutraliserend het serum is. Dit gebeurt door het mengsel op een cellaag te gieten en na een week celdood te meten, veroorzaakt door resterend virus wat niet door de antilichamen in het bloed is uitgeschakeld (plaquereductieneutralisatietest, PRNT). Om de uitslagen

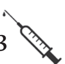
te kunnen duiden vergeleken wij de resultaten ook met 12 gezonde vrijwilligers die zich bij een van onze eerdere onderzoeken hadden laten vaccineren tegen gele koorts.

Bij geen van de patiënten deed zich een ernstige bijwerking voor. Alle patiënten lieten een beschermende antilichaamreactie zien in de gouden standaard PRNT. De resultaten van de andere laboratoriumtest (de IFA) liet slechts bij 7 patiënten een positieve uitslag zien; een opmerkelijk verschil. Bij de gezonde vrijwilligers lieten 10 van de 12 een positieve uitslag zien in de gouden standaard PRNT, en geen enkele in de IFA. Dit betekent dat de IFA slechts in 28% van de gevallen in staat is om gelekoortsantistoffen aan te tonen (de zogenaamde sensitiviteit). Een dergelijke bevinding maakt de test ongeschikt voor gebruik in de klinische praktijk voor het meten van vaccinresponsen, hetgeen geïllustreerd wordt door het feit dat twee van onze patiënten wiens immuunsysteem onderdrukt was op basis van de foutieve resultaten van de IFA opnieuw werden gevaccineerd met het gelekoortsvaccin.

Het grote verschil tussen de twee testen is niet eenvoudig te verklaren. Wellicht ligt de verklaring in het feit dat de PRNT een dynamische functionele test is waarbij meerdere componenten van het bloed kunnen bijdragen aan uitschakeling van het virus, terwijl de IFA slechts één component op een statische manier meet. Een alternatieve verklaring is een onjuiste ijking van de grenswaarde van de IFA, waarbij het testresultaat te streng wordt geïnterpreteerd. Een 10 maal mildere grenswaarde leidt tot een sensitiviteit van 67%, mogelijk ten koste van de nauwkeurigheid. Andere verklaringen worden onwaarschijnlijk geacht, zoals kruisreactiviteit in de PRNT met andere virussen van hetzelfde soort of een medicatie-bijeffect.

Uit andere onderzoeken bleek al dat patiënten met een deels onderdrukt immuunsysteem (door medicatie zoals methotrexaat) langer de tijd nodig hebben om voldoende beschermende antistoffen aan te maken. Het uiteindelijk gehalte aan beschermende antistoffen is in deze groep veelal lager dan bij gezonde proefpersonen en mogelijk houdt de bescherming dan ook korter aan. Om deze reden besloten wij om de 15 patiënten opnieuw te testen, 0 tot 18 jaar na hun eerste vaccinatie. Alle patiënten bleken nog steeds voldoende antilichamen tegen gelekoortsvirus te hebben. Voor de praktijk raden wij aan om, bij reizigers die eerder tegen gele koorts werden gevaccineerd en nu immuunonderdrukkende medicatie gebruiken, voorafgaand aan vertrek naar een gelekoortsgebied de neutraliserende antilichamen tegen het gelekoortsvirus te meten met een PRNT.

Ook in mensen met een vermoedelijk normaal functionerend immuunsysteem is het van belang om



te weten hoe lang een toegediend vaccin bescherming biedt. Dit biedt namelijk de mogelijkheid om te besparen op het aantal vaccinaties (mensen hoeven minder vaak gevaccineerd te worden), hetgeen zowel financiële als logistieke voordelen biedt, zeker in geval van beperkte vaccinvoorraden. De officiële geldigheid van de gelekoortsvaccinatie ten tijde van ons onderzoek was 10 jaar, waarna voor bepaalde reisbestemmingen revaccinatie verplicht was. In **hoofdstuk 5** onderzochten wij de duur van bescherming na vaccinatie door bij een doorsnede van 99 Nederlandse reizigers het gehalte aan beschermende antilichamen na vaccinatie in het verleden te controleren. Dit deden wij door het meten van de antistoffen met de eerder besproken PRNT en ook door het meten van immuuncellen in het bloed die direct verantwoordelijk zijn voor het bestrijden van een gelekoortsvirusinfectie in het lichaam en deze over de tijd te vervolgen.

Wij testten 99 vrijwilligers die in het verleden waren gevaccineerd, uiteenlopend van 11 tot 40 jaar geleden. In bijna 90% van hen werden mediaan 16 jaar na de vaccinatie nog steeds voldoende beschermde antilichamen gevonden. De meerderheid was 10-20 jaar geleden gevaccineerd, hetgeen direct een beperking van het onderzoek blootlegt: de conclusie over bescherming die langer dan 20 jaar na vaccinatie duurt, is gebaseerd op een relatief kleine steekproef. Het gehalte aan neutraliserende antilichamen was lager naarmate mensen langer geleden waren gevaccineerd en naarmate mensen ouder waren ten tijde van hun vaccinatie. Dit laatste fenomeen is in de literatuur onder meer ook bij vaccinaties tegen hondsdolheid en de jaarlijkse vaccinatie tegen seizoensgriep beschreven.

Het feit dat 90% van de gevaccineerde mensen in ons onderzoek veel langer dan 10 jaar na vaccinatie nog beschermd was, is aan de hoge kant vergeleken met oudere onderzoeken. Een deel van de verklaring hiervoor ligt mogelijk in de immunologische achtergrond van de vrijwilligers. Allen leefden in een rijk Westers land, hetgeen betekent dat hun immuunsysteem waarschijnlijk met minder uitdagingen te maken heeft gehad dan het immuunsysteem van iemand die in een armer deel van de wereld leeft. Zo werd in 2014 een onderzoek gepubliceerd waaruit bleek dat mensen uit Uganda na vaccinatie tegen gele koorts een lager antilichaamniveau hadden dan een qua leeftijd en ziektevoorgeschiedenis vergelijkbare groep mensen uit Zwitserland; mogelijk wordt dit verschil verklaard door een chronisch geactiveerd immuunsysteem in de eerste groep. Het is belangrijk om deze verschillen te onderzoeken en hier rekening mee te houden bij het maken van vaccinatiebeleid voor verschillende werelddelen.

In tegenstelling tot neutraliserende antistoffen na vaccinatie, is de rol van de immuuncellen die

verantwoordelijk zijn voor de directe aanval op door gelekoorts geïnfecteerde cellen (de zogenaamde CD8-positieve T-cellen) minder uitgebreid onderzocht. De manier waarop deze cellen mogelijk bijdragen aan de bescherming na gelekoortsvaccinatie is niet goed bekend. Om hier meer over te weten te komen onderzochten wij binnen dit onderzoek ook het ontstaan, de gedragingen en het verloop van deze cellen van vaccinatie tot 180 dagen nadien in een groep van 6 proefpersonen die als deel van een breder onderzoek tegen gele koorts werden gevaccineerd. Ook werden immuuncellen verzameld van 20 van de 99 vrijwilligers die in het (verre) verleden waren gevaccineerd.

De cellen voldeden niet aan het standaard functieprofiel zoals we dat doorgaans meten in het laboratorium; normaliter doorlopen deze cellen een proces waarin ze verschillende rollen spelen en waarin verschillende fasen meetbaar zijn van ‘naïef’, rijping, actieve fase en geheugenfase. Er werden veeleer cellen gezien die door alle meetpunten heen in staat waren virus te doden (actieve fase) en onderwijl ook deels veranderden in geheugencellen. Vermoedelijk wordt dit verklaard door het feit dat zelfs 6 maanden na gelekoortsvaccinatie nog steeds een actieve opbouw van de afweerreactie tegen het virus plaatsvindt. De gevonden eigenschappen en de meervoudige rollen die de aanvallende cellen leken te hebben, wijzen namelijk wél op een mogelijk beschermend effect. De betreffende cellen waren nog minstens 18 jaar na vaccinatie aanwezig in het bloed, maar vertoonden geen relatie met de hoeveelheid antilichamen in het bloed. Ook nam hun relatieve aandeel niet toe als iemand in het verleden meerdere keren gevaccineerd was tegen gele koorts.

‘Correlates of protection’

Alle testen die gebruikt worden om bescherming na vaccinatie vast te stellen in het laboratorium worden idealiter gekoppeld aan daadwerkelijk bewijs van bescherming van mensen in de praktijk. De specifieke maat die gebruikt wordt om deze bescherming in het dagelijks leven in een laboratoriumtest te vangen wordt in het Engels het ‘correlate of protection’ genoemd: een uitkomst van een laboratoriumtest die wederkerig betrekking heeft op bescherming tegen de ziekteverwekker.

Het vinden van een correlate of protection is niet zo eenvoudig als het wellicht lijkt. Vanuit wetenschappelijk oogpunt wordt het sterkste bewijs van bescherming geleverd door een provocatietest waarbij mensen het te onderzoeken vaccin of een controlevaccin krijgen toegediend en vervolgens worden blootgesteld aan de betreffende ziekteverwekker. Er wordt bijgehouden hoeveel gevaccineerden ziek worden ten opzichte van de niet-gevaccineerden, en welke merker overeenkomt met bescherming tegen de ziekte. Vaak is zo’n merker al bekend uit het verleden of uit

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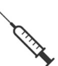
proefdieronderzoek. Doorgaans is bescherming alleen via natuurlijk verloop te onderzoeken omdat actief blootstellen aan de ziekte een onacceptabel gevaar voor de proefpersoon oplevert. Omdat de ziekten over het algemeen zeldzaam zijn is zulk onderzoek financieel en praktisch dus niet haalbaar. SARS-CoV-2 vormde hierop een uitzondering, omdat in een pandemie het aantal besmettingen erg hoog ligt en veel mensen de ziekte in hun gewone leven tegenkwamen. Voor sommige ziekten zoals malaria is het tegenwoordig ook mogelijk om op veilige wijze proefpersonen gecontroleerd actief bloot te stellen aan de ziekte. Naast bovenstaande overwegingen moet een beschermingsmaat enigszins praktisch meetbaar zijn en komt men doorgaans uit op een bekende bloedmerker zoals het gehalte antilichamen.

In **hoofdstuk 6** onderzochten we de invloed van diverse afweeronderdrukkende medicijnen van niertransplantatiepatiënten op het aanslaan van vaccinatie tegen cholera. Hiervoor werden niertransplantatiepatiënten gezocht die naast prednison slechts één ander immuunonderdrukkend medicijn gebruikten (15 patiënten per groep), om zo scherp mogelijk onderscheid te kunnen maken tussen de gevolgen van de verschillende medicijnen voor de effectiviteit van de vaccinatie. Op deze manier konden we ook de algemene mate van immuunonderdrukking van de verschillende klassen medicijnen zichtbaar maken. Nooit eerder was een choleravaccin op deze manier onderzocht in transplantatiepatiënten.

Gemiddeld reageerde 57% van de niertransplantatiepatiënten op het vaccin, tegenover 81% van de gezonde proefpersonen die als controlegroep werden gebruikt. De laagste antilichaamreactie werd gezien bij proefpersonen die het medicijn mycofenolaat gebruikten, bij hen was in 47% sprake van een afweerreactie tegen het vaccin. Dit contrasteert met 67% bij proefpersonen die een calcineurineremmer gebruiken (zoals tacrolimus). Er deden zich geen ernstige bijwerkingen voor, en het was opvallend dat de (zelfgerapporteerde) meest voorkomende bijwerking van maagdarmklachten zich meer voordeed bij gezonde proefpersonen dan bij de transplantatiepatiënten. Mogelijk vindt dit zijn oorzaak in een minder heftige reactie van het lokale immuunsysteem in de darm of omdat niertransplantatiepatiënten door hun uitgebreide medische verleden bijwerkingen beter verdragen. In eerder onderzoek met vaccinatie tegen seizoensgriep bleek al dat mensen die mycofenolaat gebruikten de laagste respons hadden op de griepvaccinatie, in die zin bevestigt dit onderzoek eerdere bevindingen. Ons onderzoek laat zien dat het wel degelijk zinvol kan zijn om transplantatiepatiënten (al dan niet oraal) te vaccineren, maar dat veel afhangt van het soort medicijnen dat een patiënt gebruikt.

Helaas was er veel variatie in de resultaten van een van de gebruikte tests om de immuunreactie aan te tonen. Dit beperkt de kracht van de conclusies en is een bekende beperking van deze test. Verder is de beste 'correlate of protection' na choleravaccinatie niet bekend: de relatie tussen gemeten antilichaamniveaus in het bloed en bescherming tegen de ziekte is niet één op één, waarbij bescherming vaak wordt onderschat door de test. Een mogelijke manier om zowel de variabiliteit als de genoemde discrepantie in de toekomst te verbeteren is het testen van antilichamen op de plek waar de cholerabacterie het lichaam daadwerkelijk plaagt en waar ook de afweerreactie het meest effectief moet zijn: de slijmvliezen van het maagdarmkanaal.



Curriculum Vitae

Emile Folkert Fermin Jonker werd op 20 mei 1987 geboren in Alkmaar, Nederland. Hij behaalde in 2005 zijn eindexamen aan het Murmelliusgymnasium te Alkmaar. In datzelfde jaar startte hij met de studie Geneeskunde aan de Universiteit Leiden. Het artsexamen werd in 2011 cum laude afgelegd. Aansluitend startte hij in 2012 met een promotieonderzoek bij de afdeling Infectieziekten van het Leids Universitair Medisch Centrum (LUMC), onder leiding van prof. dr. L. G. Visser en prof. dr. J.T. Van Dissel. De resultaten van het promotieonderzoek staan deels beschreven in dit proefschrift. In 2015 werd gestart met de opleiding tot medisch specialist binnen het vakgebied Interne Geneeskunde in de opleidingsregio Leiden, in het Haaglanden Medisch Centrum en het LUMC. Na een fellowship Intensive Care (IC) werd in juli 2022 de registratie als internist-intensivist voltooid. Aansluitend is hij begonnen aan een fellowship Acute Cardiologie in het LUMC en werkt hij ook op invalsbasis op de IC aldaar.

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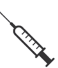

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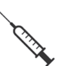

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